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Assays of Toxic Pollutants by Fish Blood

Sias Research Labs, Brookline, Mass

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We have investigated and recorded the response of F. heteroclitus whole blood cells from fishes living in several aquatic environments of fixed pH and dissolved oxygen and temperature. We compared these data with those obtained from fish subjected to dissolved traces of chemical pollutants. In final fullfillment of our grant, we have delivered an advanced model of the multichannel analyzer to the U. S. Water Quality Laboratories in Narragansett, Rhode Island. The Fish Blood Analyzer is produced by Grumman Health Systems.

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ASSAYS OF TOXIC POLLUTANTS BY FISH BLOOD

by

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CONTENTS

Section		Page
I	Introduction	1
II	Materials and Methods: General Test Procedures	9
III	Results: Controls	12
IV	Results: Freshwater Adaptation Experiments	15
V	Results: Cadmium Experiments	23
VI	Results: Copper Experiments Freshwater vs. Saltwater	28
VII	Results: Copper Experiments 5ppm and Recovery Tests	40
VIII	Discussion	57
IX	Bibliography	65

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FIGURES

		Page
1	Biological Mulitchannei Analyzer	5
2	Analyzer Storage Output from Storage	6
3	Sensor and Secondary Pulses Representative Pulse Shape	7
4	Triggering Delays Electronic Pulse and Cell Shape	8
5	Block Diagram of Multichannel Analysis System for Fish Blood	62
6	Block Diagram of GHS Fish Blood Analyzer	63
7	GHS Fish Blood Analyzer	64

TABLES

		Page
I	Fish in $CdCl_2$ and Reversed	24
II	Fish in $CdCl_2$ and Reversed: By Channel (Peak) Number	25

ILLUSTRATIONS AND PHOTOGRAPHS

	Page
Photomicrograph: Control	13
Electronic Printout: Control.	14
Photomicrograph: Freshwater 2, 4, 6 Hours and Phase-over	18
Electronic Printout:Freshwater 4 Hours and Phase-over	19
Photomicrograph: Freshwater Adapted	20
Electronic Printout: Freshwater Adapted	20
Photomicrograph: Distilled Water 1, 3, 4 Hours	21
Electronic Printout: Distilled Water 1, 3, 4 Hours	22
Photomicrograph: $CdCl_2$ 3.5 and 5.75 Hours	26
Electronic Printout: $CdCl_2$ 3.5 and 5.75 Hours	27
Photomicrograph: $CuCl_2$ in Freshwater and Saltwater 24 Hours, 1.5ppm	31
Electronic Printout: $CuCl_2$ in Freshwater and Saltwater 24 Hours, 1.5ppm	32
Photomicrograph: $CuCl_2$ in Freshwater and Saltwater 96 Hours, 1.5ppm	33
Electronic Printout: $CuCl_2$ in Freshwater and Saltwater 96 Hours, 1.5ppm	34
Photomicrograph: $CuCl_2$ in Freshwater and Saltwater 1 Hour, 2ppm	35
Photomicrograph: $CuCl_2$ in Freshwater and Saltwater 3 and 5 Hours, 2ppm	36
Electronic Printout: $CuCl_2$ in Freshwater and Saltwater 1, 3 and 5 Hours, 2ppm	37
Photomicrograph: $CuCl_2$ in Freshwater and Saltwater 24 Hours, 2ppm	38
Electronic Printout: $CuCl_2$ in Freshwater and Saltwater 24 Hours, 2ppm	39
Photomicrograph: $CuCl_2$ in Saltwater 45 Minutes, 5ppm	46

	Page
Photomicrograph: $CuCl_2$ in Saltwater 1Hour 3ppm 4.5 Hours, 5ppm	47
Electronic Printout: $CuCl_2$ in Saltwater 4.5 and 5 Hrs 5ppm	48
Photomicrograph: $CuCl_2$ in Saltwater 24 Hours, 5ppm	49
Photomicrograph: $CuCl_2$ in Saltwater 44 Hours, 5ppm	50
Electronic Printout: $CuCl_2$ in Saltwater 24 and 44 Hours	51
Photomicrograph: $CuCl_2$ in Saltwater 24 Hours, 5ppm Returned to Unpolluted Saltwater 10 Hours	52
Photomicrograph: $CuCl_2$ in Saltwater 24 Hours, 5ppm Returned to Unpolluted Saltwater 1 Week	53
Electronic Printout: Return 10 Hours Return 1 Week	54
Hypothetical Model: Effects of Cu^{++} Exposure on Fish Erythrocytes	61

INTRODUCTION

Fundulus heteroclitus exhibits rapid physiological responses to changes in environmental conditions. We have been particularily interested in the ability of the peripheral blood cells to cope with various environmental insults, i.e. changes in salinity and exposure to heavy metals. In studying the responses of the blood cells, we have examined the cell populations by classical methods such as microscopic study of both fresh and Wright stained preparations. More importantly, we have examined the blood cell populations using a Biological Multichannel Particle Size Analyzer (BMA). The BMA electronically monitors the cell population, giving a size and frequency distribution. Our tests have confirmed our ability to use this electronic assay system to pick up the morphological changes that are seen in the photomicrographs of both the phase contrast studies and the Wright stained cells. The importance of using a multichannel analyzer, such as the BMA, to detect changes in the blood cell population lies in its rapidness and sensitivity. The BMA can sample within seconds a statistically more appropriate number of cells than more classical methods of differential counts from slides. Further, the BMA can indicate changes in blood cell morphology, as well as shifts in the composition of the components of the blood cell population. These changes are indicative of the sublethal effects of an environmental pollutant. The sublethal effects of a pollutant may be just as damaging to the continuing exsistence of a population as an outright fish kill. The impairment of breeding potential, the greater vulnerability to other environmental stresses, and the reduced viability of the offspring, all these factors conspire in diminishing the population's ability to florish or even to survive in its natural habitat.

The Biological Multichannel Analyzer (BMA)

In the search for more rapid and accurate methods for studying the dynamics of cellular growth and changes and rates of changes in sizes, shapes and numbers of cells in living populations of organisms, we developed a system built around a transducer operating on the differential conductivity principle of Coulter. Through proper interfacing, we have matched the particle sensor to a 200 multichannel analyzer. The prototype BMA system is presently in use at the Naval Biomedical Research Laboratory, Oakland, California. An advanced model of the system is at the Naval Weapons Laboratory, Dahlgren, Virginia.

The BMA system is computer compatible and capable of rapidly defining the size and frequency distribution of a population of organisms and particulate material contained in fluid media. The system can display population distributions over size ranges of interest to us on a minute by minute basis if desired.

Figure 1 shows the analysis unit for the studies to be reported here. The sensor is a manometer element of the type used in the Coulter particle counter, and operates on the Coulter principle viz.,

particles are carried in a conductive fluid through an aperture through which an electric current is flowing. Each time a particle passes through the aperture, the current flux density is altered in the aperture. This alteration in flux density causes a change in potential across the aperture. The change in potential is amplified and measured in subsequent circuitry. The resulting output from the sensor during the passage of several spherical particles moving at a constant flow velocity (controlled in the system) produce a series of voltage pulses which are approximately 20 microseconds in duration and have an amplitude which is proportional to the size of the particles.

Under constant flow rate conditions, it is possible to measure and record pulses generated by particles in fixed intervals of time. The equipment can store the information from one sample of fluid at a time within its core memory and must be emptied after a digital or analog form representation of the data contained in the memory has been made. It is possible, however, in more complex equipment to produce a duplicate of the data going into the core storage in the memory bank of a time-shared computer or to record the data on magnetic tape. The system couples the Coulter sensor through the proper interfacing into a Nuclear-Chicago 200 Channel Multichannel Pulse Height Analyzer. The pulses produced by the sensor are amplified and fed into a secondary pulse generator which produces fixed duration uniformly shaped pulses proportional in height to the input pulses. These pulses are measured and assigned a position on the X axis of the analyzer. This axis is divided into 200 parts or channels. Each time a pulse of the same height is measured, one more number is added to that particular channel. Thus, the storage will show the number of pulses (particles) which have been assigned to a given channel at any instant in the sampling period. At the end of the sampling period, the storage will contain the total count (P_x) for pulses having any given pulse height within a pulse height range selected by the operator to be contained within the 200 channels of the storage. The data included in this report have been recorded in analog form using a Mosley X-Y plotter.

In Figure 2, we have attempted to clarify the concept by showing a representation of what information would be contained in the first 100 channels of storage for a hypothetical sample. The stored information would be the digital record of the number represented as equal blocks in each channel, as shown in the upper graph. The analog output from storage would be made by reading out a voltage proportional to the number of blocks in each channel starting at channel one and ending with channel 100. The completed read-out as made on the X-Y Plotter is shown in the lower graph. It should be noted that in the lower graph the ordinate and abscissa definitions have changed. The Y axis reads the particles counted since there is a one to one relationship to the pulses generated by the sensor provided certain conditions are met. The X axis absolute scale will change depending on the values of the control parameters set into the system. For any operating conditions set into the equipment, a calibration curve is made using latex spheres of known sizes as standards.

For ease of comparison, we calibrate on the basis of particle diameter rather than volume. A calibration on the basis of volume is possible whenever it is desired.

Total counts can be obtained from the X-Y plots using the following relationship:

$$P = S \sum_{x_{min}}^{x_{max}} P_x \Delta x$$

Where P = the total particle count

P_x = the particle count per x increment

and

S = scale factor (as set within the equipment)

The x increment is usually taken as the width of one channel, in which case for dilute solutions the integration constant may be taken as unity. x_{min} and x_{max} are determined by the region of interest, $x_{min} \geq 0$ and $x_{max} \leq$ maximum number of channels in a memory block.

Because of the properties of the fish blood, pulse shape analysis must be done. The system of pulse height analysis using the Coulter transducer requires for proper operation, that the flow rate through the counting aperture be absolutely precise. This means that the durations of pulses generated by spheres of equal size will be equal. The 30 micron aperture wafer thickness is usually 40 - 50 microns. The flow rate which is normally available using the Coulter manometer with a 30 micron aperture is such that pulses of 20 microseconds duration (base to base) are generated when 1 micron spheres traverse the aperture. The geometry and flow rate characteristics are such that pulses of the same order duration are produced by 2.7 micron diameter spheres passing through a 100 micron aperture.

The pulses produced by spheres have bilateral symmetry peaking at 10 microseconds. The BMA equipment is normally set to trigger the production of a 2 microsecond square wave pulse having an amplitude which is linearly proportional to the voltage of the pulse produced by the sensor at the exact instant of the triggering (Figure 3).

As the diameter of the sphere increases, the pulse duration increases, but with a much smaller magnitude than the pulse amplitude at the gain settings normally used for routine analysis.

Figure 3B shows the type of pulse normally seen when samples of human erythrocytes are diluted on 0.9% physiological saline (particle-free) and passed through a 100 micron aperture. The resulting pulse train is made up of a population of pulses which differ from one another only

slightly. With the BMA equipment set to trigger at 8 microseconds into the pulse (DM = 8 mocroseconds), secondary voltage pulses of amplitudes proportional to the sensor voltage at point "a" will be produced. If the delay trigger is set to produce a secondary pulse 25 microseconds after the initiation of the sensor pulse, pulses proportional to the sensor voltage at point "b" will be produced. It will be noted that due to the fact that uniform pulses are being produced by the sensor, the resultant change in the delay of secondary pulse triggering results only in a shift of the apparent diameter to the right ($a \rightarrow a'$ in Figure 4A).

This change in the apparent diameter is artificial since the size index of a particle (be it diameter, volume, cross-sectional area or any other dimension upon which a calibration curve was produced from a known particle population calibration standard) will be different for each delay trigger setting. In the data herein presented, all size calibration data are based on an 8 microsecond delay and all X axis scales are given relative to the DM 8 microsecond calibration value. In practice, reference of pulse distributions to a single trigger delay is useful since the changes in size indices of particles giving characteristic complex pulse shapes will be reflected in shifts in the BMA plotted size distributions from those produced by the population of the same component particle mixes prior to changes in the size indices. Since the assay data must be based upon differences from normals, and since the normals are catalogued emperically, the system requires only absolute precision to be effective. The system does have the requisite precision for its utilization as an extremely sensitive differential pulse shape analyzer.

Interpretation of shape by two point (or three point) variation of trigger delay can be made only if an analysis of the shapes of the pulses in the array being studied can be made. We have made subjective observations of the sensor pulse arrays and have observed at least three generally occurring pulse shapes. Figure 4B illustrates these and indicates the probable form of the particles producing each.

The power of this equipment lies in its high sensitivity (changes as small as 10 cells/sample volume of 0.05ml can be easily seen), precision and rapid analysis interval (less than 15 seconds/sample actual sample analysis and storage time).

Figure 1

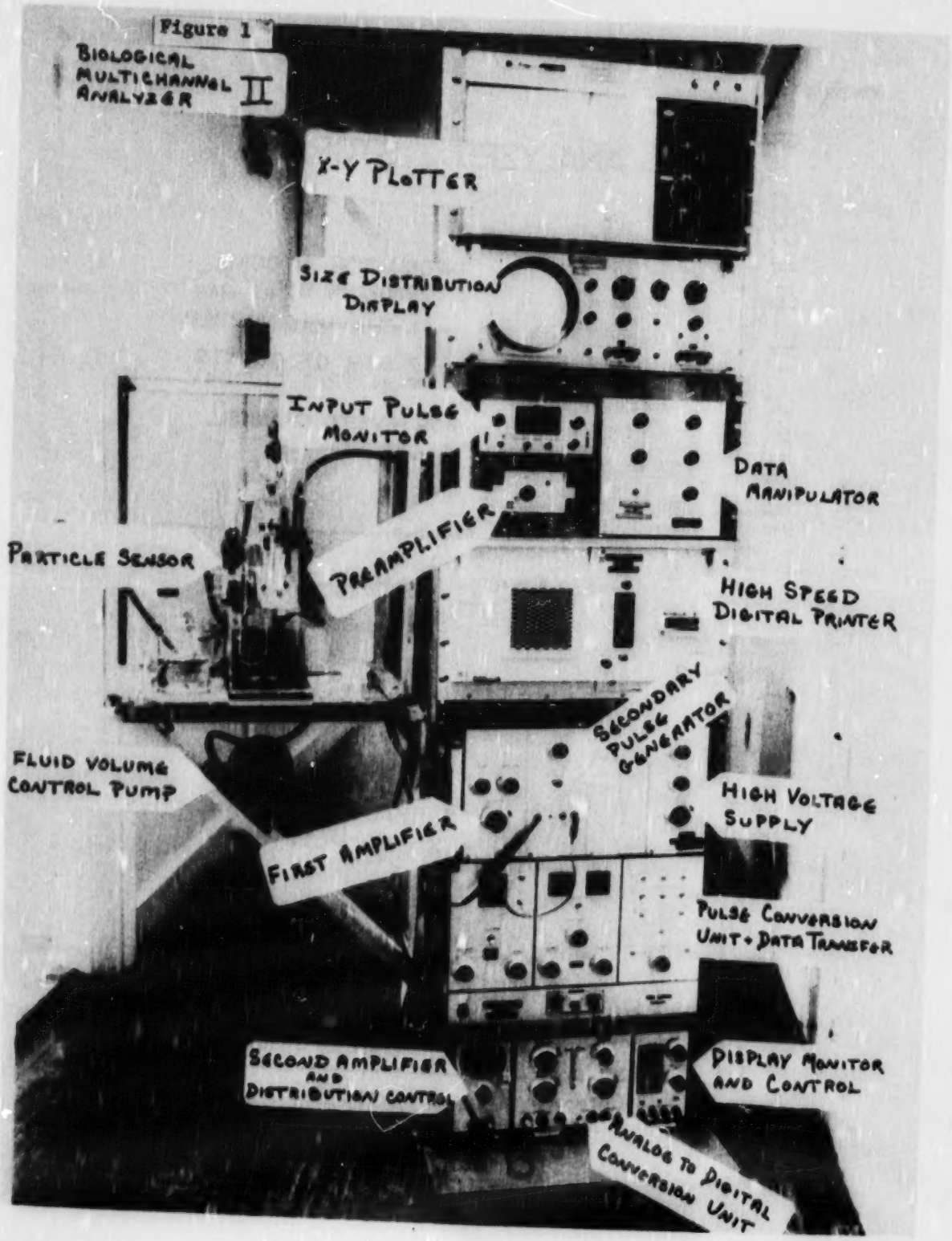

BIOLOGICAL MULTICHANNEL ANALYZER II
X-Y PLOTTER
SIZE DISTRIBUTION DISPLAY
INPUT PULSE MONITOR
DATA MANIPULATOR
PARTICLE SENSOR
PREAMPLIFIER
HIGH SPEED DIGITAL PRINTER
SECONDARY PULSE GENERATOR
FLUID VOLUME CONTROL PUMP
HIGH VOLTAGE SUPPLY
FIRST AMPLIFIER
PULSE CONVERSION UNIT + DATA TRANSFER
SECOND AMPLIFIER AND DISTRIBUTION CONTROL
DISPLAY MONITOR AND CONTROL
ANALOG TO DIGITAL CONVERSION UNIT

FIGURE 2

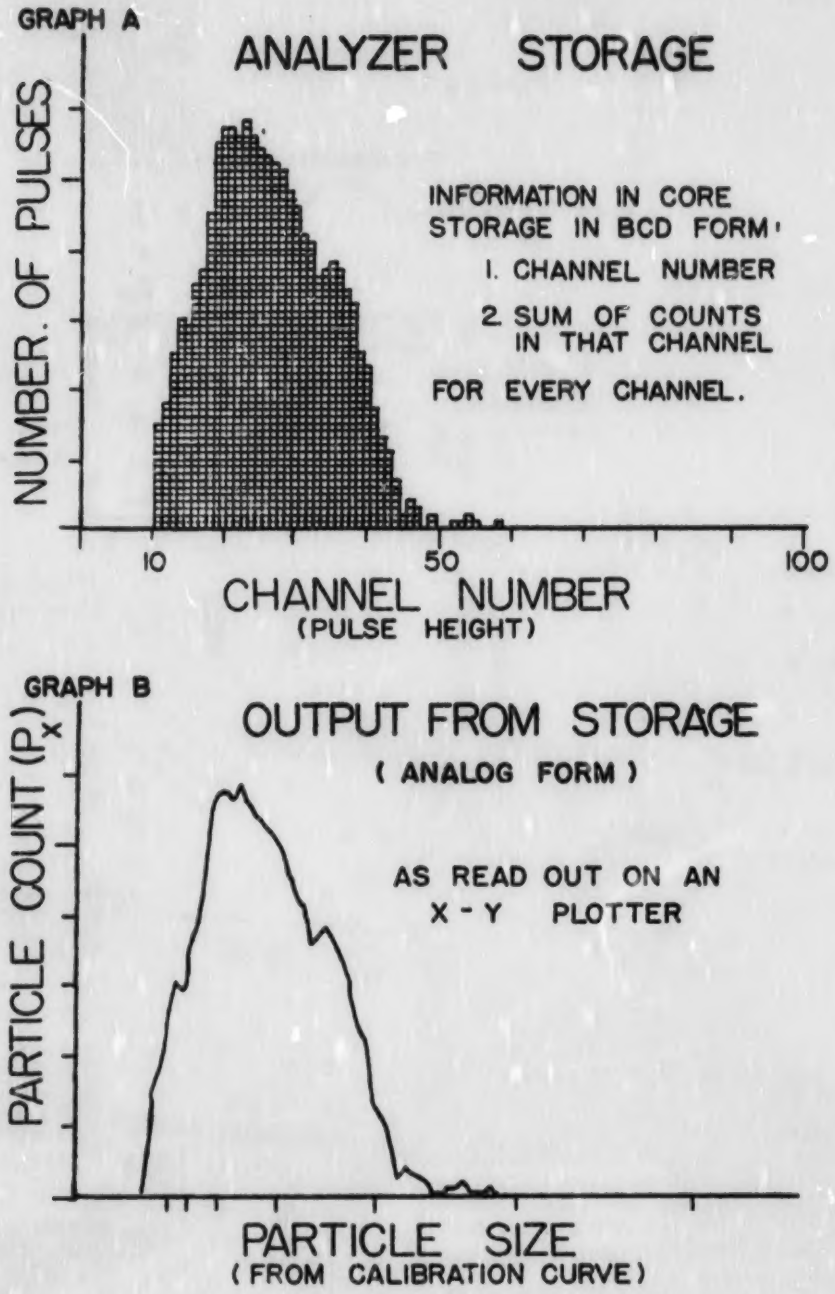
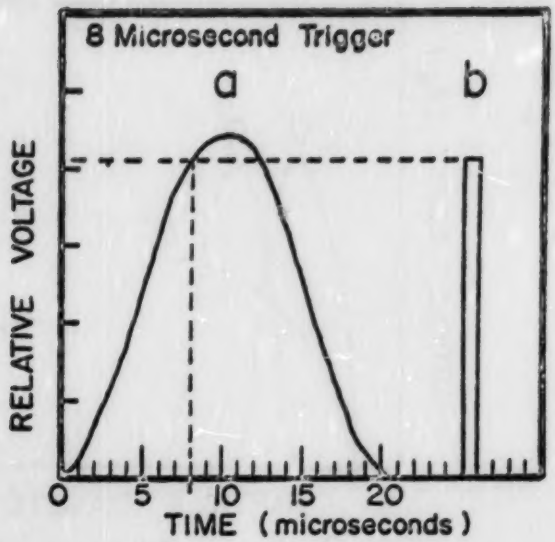

GRAPH A
ANALYZER STORAGE
NUMBER. OF PULSES
INFORMATION IN CORE STORAGE IN BCD FORM:
1. CHANNEL NUMBER
2. SUM OF COUNTS IN THAT CHANNEL
FOR EVERY CHANNEL.
10
50
100
CHANNEL NUMBER
(PULSE HEIGHT)
GRAPH B
OUTPUT FROM STORAGE
(ANALOG FORM)
AS READ OUT ON AN X - Y PLOTTER
PARTICLE COUNT (P_x)
PARTICLE SIZE
(FROM CALIBRATION CURVE)

FIGURE 3

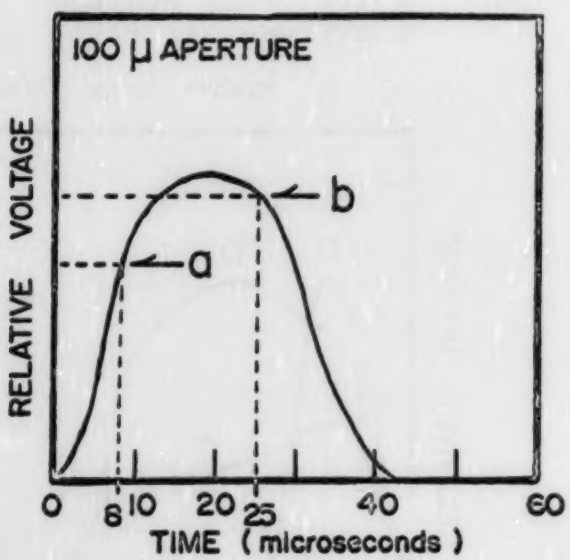
A



PULSE a = SENSOR PULSE

PULSE b = SECONDARY PULSE

B



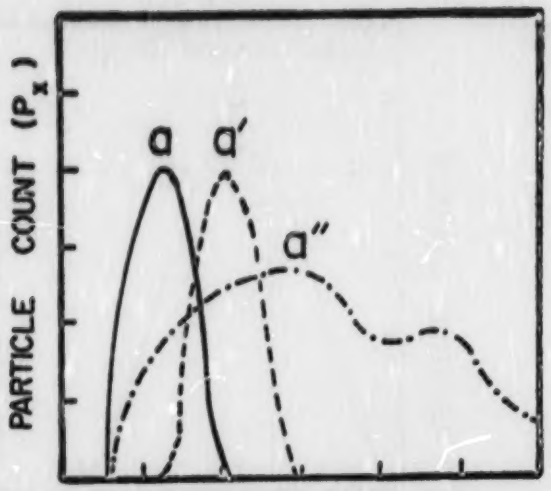
REPRESENTATIVE PULSE SHAPE :
HUMAN ADULT MALE ERYTHROCYTE
(In 0.9 % w/v Saline)

a. 8 Microsecond Delay Trigger

b. 25 Microsecond Delay Trigger

FIGURE 4

A

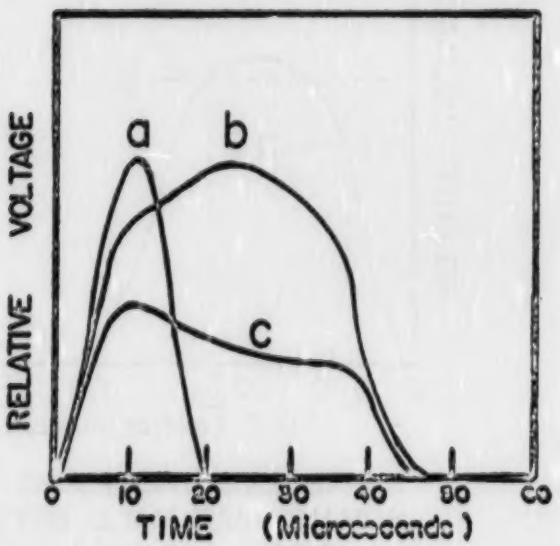


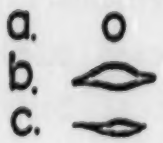
— a. 8 usec. Trigger

-- a'. 25 μsec Trigger on a Symmetrical Highly Uniform Pulse Population

-·-a" 25 μsec Trigger on a Semi-random Mixed Pulse Population

B





MATERIALS AND METHODS: GENERAL TEST PROCEDURES

Daily Maintenance

The populations of Fundulus heteroclitus used in these experiments were obtained from the Environmental Protection Agency headquarters in West Kingston, Rhode Island. They were caught offshore of Naragansett Bay just prior to their transportation to our laboratories in Brookline, Massachusetts. They were transported in large plastic containers fitted with air bubblers. The two hour trip never affected the fish adversely.

The Fundulus population was maintained in 50-gallon glass tanks filled with artificial seawater (Triton)*. The water was filtered continuously through polyester fiber in Dynaflo water filters**. The water was aerated by plastic bottom filters attached to an air pump with a non-oil compressor. Plastic cooling coils kept the temperature of the tanks at 17°C (±0.5°C). The water running through the coils was cooled by a large Thermovac refrigeration unit***.

The pH of the water, buffered wuth $NaHCO_3$, was kept at 6.8 (±0.2). The temperature of the tanks was monitored continuously by Weather-Hawk recording thermometers****. Other parameters measured included specific gravity, room temperature, relative humidity, and osmolarity. The fish were fed once daily with frozen brine shrimp.

The fish kept in the tanks at our laboratory seemed quite healthy. They ate well, swam through the tank energetically, and on only one occassion were they affected by external parasites or fungus. The male fish went into their breeding colors - the black spot on the dorsal fin, the silver striping on the sides, and the yellowish belly. The popula- of Fundulus carried over the winter and did reproduce in the spring. However, the offspring did not survive; this is not surprising considering the number of fish present in the large holding tanks.

Freshwater Experiments

Since F. heteroclitus is a euryhaline fish and there had been problems with metals precipitating out in saltwater, it was decided to adapt some of the saltwater fish to a freshwater environment. It was found at the beginning that putting fish directly into freshwater from the saltwater tank caused an 100% mortality within a few days. Subsequently, a phase-over brackish tank (14‰) was used to lessen the physiological shock of the transfer.

Specifically, a 20-gallon tank was filled with artificial seawater

* Aquarium Systems Inc., Eastlake, Ohio
** Metaframe Corp., Maywood, New Jersey
*** Thermovac Industries Corp., Copiaque, New York
**** Taylor Instrument Companies, Asheville, North Carolina

in half the concentration of the saltwater control tank. This tank was buffered with $NaHCO_3$ to a pH of 6.8 (±0.2). There were no cooling coils in this tank, but the slightly higher temperature did not seem to bother the fish, as there were no mortalities. After a minimum of one week in the brackish tank, the fish were transferred to a 50-gallon freshwater tank. This tank was equipped with cooling coils. With the initial phase-over into brackish water, the fish, though obviously stressed in the freshwater, did not show the high mortality seen previously.

Freshwater experiments were designed to indicate the degree of adaptation to the new environment. Fish from the phase-over 14‰ salinity tank were sacrificed after a week in that environment. Similarily, after a minimum of a week in the freshwater tank, fish were sacrificed to examine the condition of the circulating blood. The success of this phase-over method of adaptation can be seen by comparison with the experiments done placing fish directly into freshwater

Copper and Cadmium Experiments

Tests with Cu^{++} and Cd^{++} consisted of both short-term, long-term, and recovery experiments. Metal ion tests were conducted in both freshwater and saltwater. The concentrations were kept sublethal (5), no more than 5ppm for either Cu^{++} or Cd^{++} was used.

The primary test involving Cd^{++} was a chronic exposure to 1ppm for ten months. The fish were maintained at the EPA headquarters in West Kingston, R.I., and were brought up to these laboratories for testing. Some of these fish were placed back in unpolluted saltwater for periods of time from 21 - 42 hours, and then sacrificed.

The Cu^{++} tests were conducted in both freshwater and saltwater. Test fish were placed in 1-gallon glass jars equipped with air bubblers. Observations of fish behavior and appearance were made throughout the exposure period. A stock solution of $CuCl_2$ was made up so that an accurate measure of the concentration in the test jar could be made. The experimental jars were monitored with a Lamont Copper Test Kit*. In the recovery experiments, the fish were put into 20-gallon saltwater tanks after their exposure to Cu^{++}.

The 1-gallon glass jars in these experiments were filled and then immersed in a 15-gallon tank. Since the tank was equipped with cooling coils, by immersing the jars in the tank, the water in the experimental jars could be maintained at the temperature of the control tank. After the experiments, these jars were cleaned thoroughly.

* LaMotte Chemical, Chestertown, Maryland

Biological Multichannel Analyzer (BMA) and Photomicroscopy

At the time of sacrifice, fish were selected at random from the saltwater control tank or the experimental jar/tank. As rapidly as possible, the fish was sacrificed by making a transverse cut two-thirds of the body length back from the head. Blood was collected from the caudal artery in heparinized capillary tubes. One sample was put into 50ml 0.9% particle-free physiological saline to be monitored on the BMA. Other samples went onto microscope slides for phase contrast observations and for Wright staining.

The sample diluted in 50ml physiological salin was monitored on the BMA as soon as possible after the sacrifice of the fish, usually within a minute. The technical explanation of the mode of action of the BMA is found in the Introduction. Using a sensor with a 30μ aperture, 0.05ml from each sample was read at 4, 8 and 25μsecond triggering delay intervals. A printout was made of each distribution for the three triggering delay intervals. A peak channel was determined, and the total cells sampled were recorded using the 8μsecond delay setting. The BMA was calibrated using a variety of polystyrene spheres and plant pollens on the 8 second delay setting. In this way, the printouts could be interpreted by correlating channel number with cell width in microns. The lower limit of the printout was estimated at 3μ, the upper limit at 10μ. Human blood cells, which are 5 - 6μ in diameter, when monitored on the calibrated electronic settings for fish blood came up midway in the 0-200 channel printout range. This is another indication of the correctness of our estimated limits.

The slides of the blood cell samples made up for photomicroscopy were allowed to air-dry. In the latter part of this research, phase contrast microscopy was done in conjunction with the Wright staining. Slides were Wright-stained using a commercial kit made by Gugol Clini-Tex, Inc.* The stained cells were examined under oil immersion at 930X. Photomicrographs were made of representative fields; the negatives were made into 5x7 prints. Measurements of cell length and width, and nucleus length and width were made from the prints. The measurements were analyzed statistically to obtain averages, variance, standard deviation, and standard error. Calibrated polystyrene latex spheres were used as size references, and a stage micrometer was used to obtain size information on the cells studied under the microscope. Calibrated photomicrographs were also made of the cells under phase contrast microscopy.

* Gugol Science Corp., North Elmsford, New York

RESULTS

Controls

Specimens taken from the saltwater holding tank at random were sacrificed during each experiment to check the stability of the fish population.

Photomicrographs:

The circulating blood of F. heteroclitus is composed predominantly of mature nucleated erythrocytes. These erythrocytes are elliptical in shape with a centrally located nucleus. The fixed erythrocytes measure between 10μ and 11μ in length, and 5μ to 7μ in width.

Phase contrast optics indicate a smooth erythrocyte membrane (page 13) Round dense granules appeared in many cells on the inner edge of the cytoplasmic membrane. The nuclei of the erythrocytes under phase contrast optics were of an oval shape. The nuclei were dense and concentrated in the center with a less dense nuclear membrane.

The photomicrographs of the Wright stained cells reveal the uniform size and regular shape of the erythrocytes (page 13). The cytoplasm of the erythrocytes stained pale pink, the nuclei stained a dark magenta. Granules were present in some cells on the inner edge of the cytoplasmic membrane. The nuclei of these cells consist of densely packed and darker stained chromatin clumps.

Electronic Printouts:

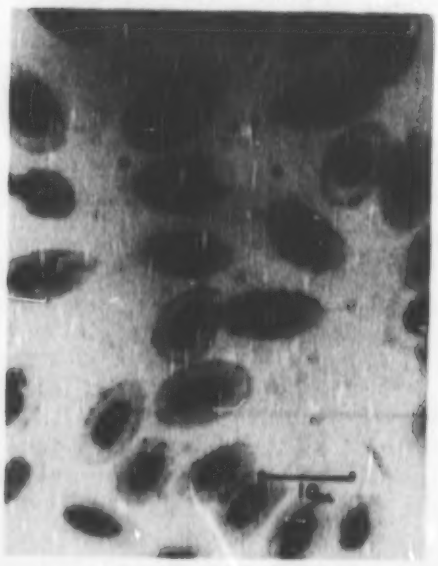
The BMA printouts of the saltwater control cells have shown a consistent picture of normal population distributions (page 14). The three triggering delay intervals each have a distinct curve as well as a specific relationship with one another. Generally, the 4μsec triggering delay interval was a single steeply risng and falling curve. The distribution of the 8μsec triggering delay interval had a primary peak which fell slightly and then rose again to a smaller secondary peak; this interval had a broader distribution than the 4μsec interval. The 25μsec triggering delay interval had the broadest of the three distributions. Like the 8μsec interval, the 25μsec interval had primary and secondary peaks in approximately the same positions as the 8μsec interval. In the control population, the peak of the 4μsec interval and the primary peaks of the 8μsec and 25μsec intervals all overlapped each other.

Just as there are fairly specific parameters for the normal population as described above, there are also certain common deviations seen in an abnormal experimental situation. In an abnormal population, instead of overlapping one another, the peaks of the three triggering delay intervals will separate from each other. As the peaks begin to shift apart, there is an associated reduction and finally disappearance of the secondary peaks of the 8μsec and 25μsec triggering delay intervals. We have been able to associate this peak shifting and peak reduction with changes in the integrity of the cell membrane as well as changes in the proportions of cell types in the circulating blood of chemically insulted fish.

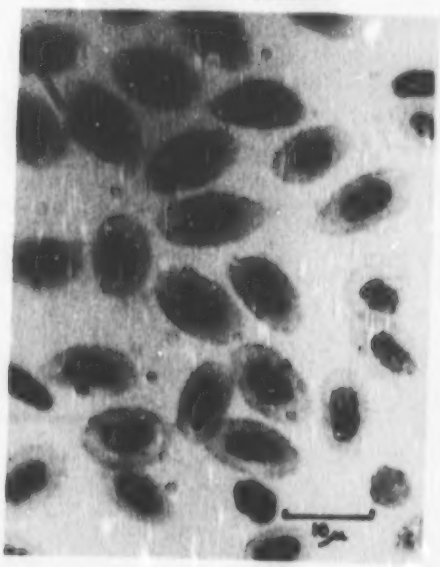
CONTROL: 5/15/73 PHASE

CONTROL: 5/15/73 PHASE

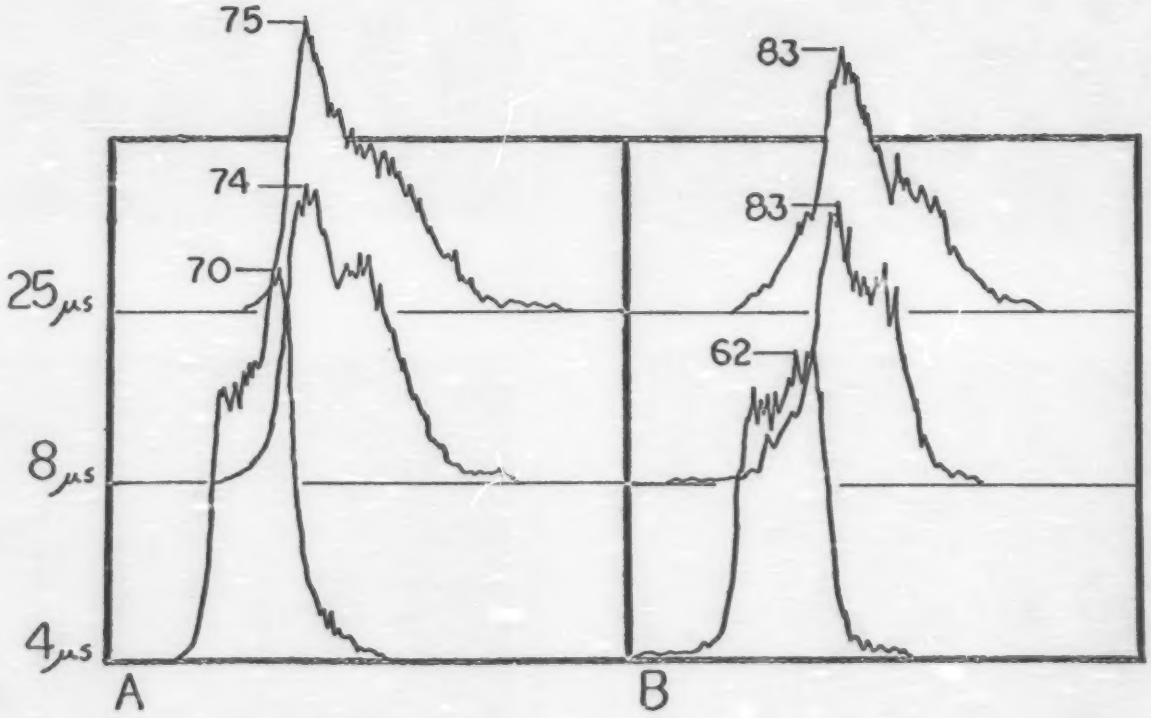
CONTROL: 5/21/73 STAINED



CONTROL: 5/21/73 STAINED



CONTROLS



RESULTS

Freshwater Adaptation Experiments

The freshwater experiments provided information on the ability of Fundulus heteroclitus to cope with an environmental stress for which it has the physiological capacity to adapt to. The osmotic stresses of a freshwater insult were easier to interpret than the more complicated actions of metal ions on the circulating blood.

A variety of adaptation tests were conducted - short-term tests in which the fish were in freshwater for less than 24 hours, longer tests lasting up to one week, and extended tests in which the fish were freshwater adapted for over one week. Our objective in these tests was to determine how long it took a population of F. heteroclitus to overcome the initial stresses of the freshwater environment. Adaptation was evaluated by 1) an appraisal of the cells seen in the photomicrographs including the statistical information from the photomicrographs, 2) a determination of the extent to which the electronic printouts resembled the printouts of the control fish, and 3) the judgement of an experienced observer of a return to more normal behavior and appearance of the adapted fish.

The first freshwater experiments were done by placing the fish directly from the saltwater tank into buffered distilled water. This change was too abrupt and would have resulted in 100% mortality within a few days if the fish had not been sacrificed. The fish displayed common stress reactions: remaining motionless at the bottom or very top of the tank, swimming on the side or belly-up, and finally hemorrhaging around the mouth and gills followed within hours by death.

The extreme reaction caused by the abrupt environmental change was compared to a gradual phase- over of the fish from saltwater to freshwater. As described in the Methods section, a 14‰ salinity tank was set up to reduce the stress of the fish. The fish remained in this tank for a minimum of a week before being placed in the freshwater tank; there were no mortalities in the 14‰ salinity tank.

None of the fish put into the freshwater tank from the 14‰ salinity tank showed any signs of internal hemorrhaging, though a certain number died during the first week, which was the most critical period. A number of common stress reactions and behavior patterns were observed to be exhibited by the fish in the freshwater. The first reaction of the fish was to sink to the bottom of the tank and remain nearly motionless; a smaller number would float near the surface, also remaining motionless. Any swimming was feeble and only for short periods of time, there was none of the constant swimming about throughout the tank that is characteristic of normally active fish. When being fed, the fish had no sense of the food in the water; they did not swim up to the surface when fed. Very often, there were color changes in the fish, many becoming splotchy with

white spots on the darker body.

After about a week the fish appeared more normal in behavior and appearance. They swam freely throughout the levels of the tank. Before feeding, the fish swam to the front of the tank seemingly in anticipation of being fed. This reaction has been observed often and was one indication of a healthy fish. After 2-3 weeks in the freshwater tank, the electronic tests conducted indicated that the circulating blood in these outwardly healthy fish was similar to that seen in the controls.

After 3-4 weeks in the freshwater tank, some fish were tested by placing them in jars of fresh buffered distilled water. We wished to determine whether the fish were really adapted to the freshwater or whether the fish had changed some chemical or physical properties of the freshwater they were in that eased their adaptation and resulted in nearly 0% mortality. If the fish were again put into fresh distilled water, would they exhibit the same stress reactions and greater mortality?

Photomicrographs:

Phase contrast microscopy was not done at the time of these experiments.

The Wright stained photomicrographs of the fish placed directly in freshwater without a gradual phase-over reveal striking abnormalities from the normal blood picture. The cells became swollen and in many instances the cytoplasmic membrane broke down leaving enlarged free nuclei (page 18). Another distinct feature was the large per cent of thrombocytes in the circulating blood. Thrombocytes are involved in the clotting process in fish. Since the fish showed signs of hemorrhaging around the mouth and gills, it was not surprising to see the great number of thrombocytes. These cells may join to form a thrombocytic network which is formed by the cohesive properties of the cytoplasm of the thrombocytes.

The photomicrographs also indicated an altered permeability of the cell membrane to the Wright stain. The cells of the fish exposed to freshwater for short periods of time (up to 2 hours) did not take up the stain normally (page 18). The nuclei were indistinct, rather than appearing dense as they usually do. The longer the fish remained in the freshwater, the more distinct the nuclei appeared in the photomicrographs. To explain this change in permeability, it was theorized that during the first few hours in freshwater, the cells are undergoing either partial or total hemolyzation due to the changes in the osmotic pressure. It was this process which affected the cell membranes.

During the gradual phase-over to freshwater, fish were sacrificed after a minimum of one week in the 14‰ salinity tank. The cells in the photomicrographs were not as uniform in size and shape as the control cells, but otherwise they appeared normal, i.e. there was no swelling and there were a normal number of thrombocytes (page 18).

After 8-10 days in the freshwater tank, the fish appeared much less stressed, and some of the fish were sacrificed to try to determine the degree of adaptation. The cells measured in the photomicrographs differed from the control cells in that there was an average increase of 1μ in the length of the freshwater adapted cells. There were few free nuclei and an average number of thrombocytes (page 20).

Finally, when fish were put into the fresh distilled water from the freshwater tank, the cells and nuclei became swollen in the first hour (page 21). As the exposure was continued, the cells and nuclei became less swollen in all dimensions and the variance decreased (page 21).

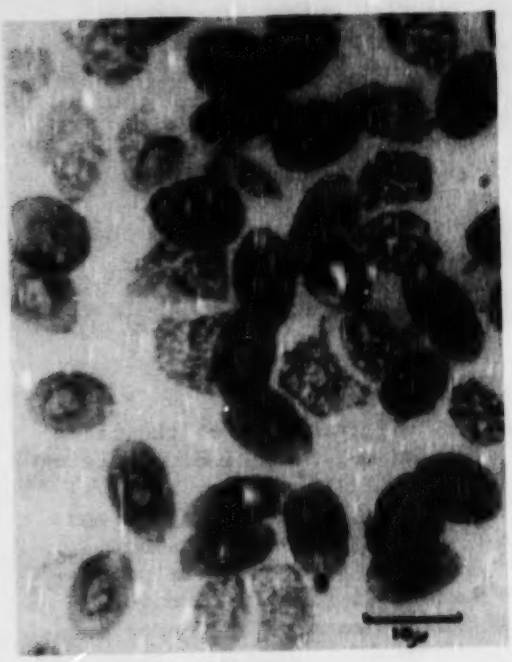
Electronic Printouts:

There are several features that distinguish the printouts of the cells of fish placed directly into freshwater with no phase-over period. There was often a large pre-peak build up in the 4 sec triggering delay interval (page 19). The build up occurred from the lower limit of the printout and merged with the primary peak. The size range of this large population was 3-4 microns in width; this probably represented the thrombocyte population seen in the photomicrographs. Another feature of these printouts was the reduction or disappearance of the secondary peaks of the 8μsec and 25μsec triggering delay intervals.

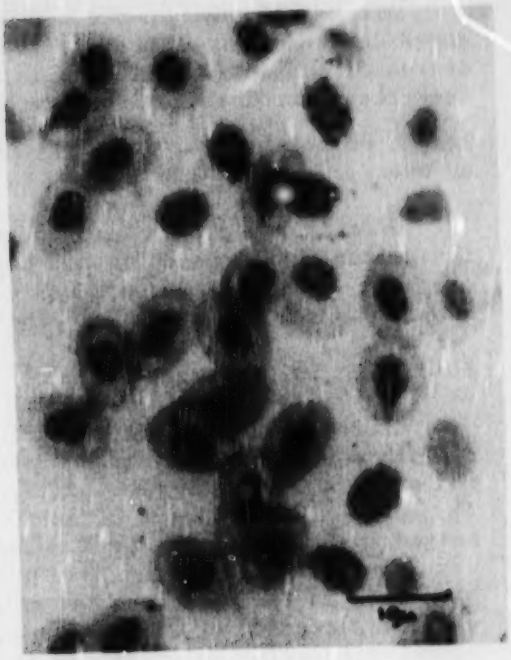
Fish sacrificed from the 14‰ salinity phase-over tank had printouts resembling the controls (page 19). The distribution of the 4μsec triggering delay interval was more spread out than the control; this indicated that there was quite a variance in the cells seen at this interval. The 8μsec and 25μsec triggering delay intervals were fairly normal, both intervals having some distinguishable secondary peak.

The fish adapted to freshwater were much more normal than those fish placed directly in freshwater with no phase-over, in terms of what the electronic printouts revealed (page 20). In the 4μsec triggering delay interval there was a slight pre-peak build up and the distribution was quite broad; but the deviation from the control situation was not so obvious. The 8μsec and 25μsec triggering delay intervals were both normal.

It appeared from these freshwater experiments that the changes which occurred due to exposure to freshwater were reflected primarily in changes in the distribution made at the 4μsec triggering delay interval.

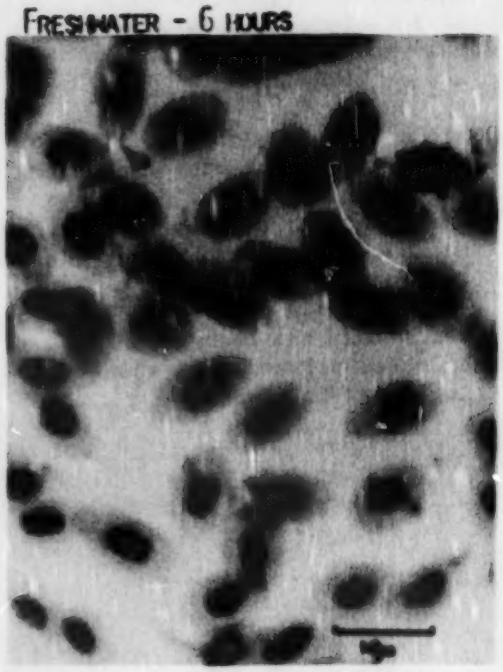


FRESHWATER - 2 HOURS

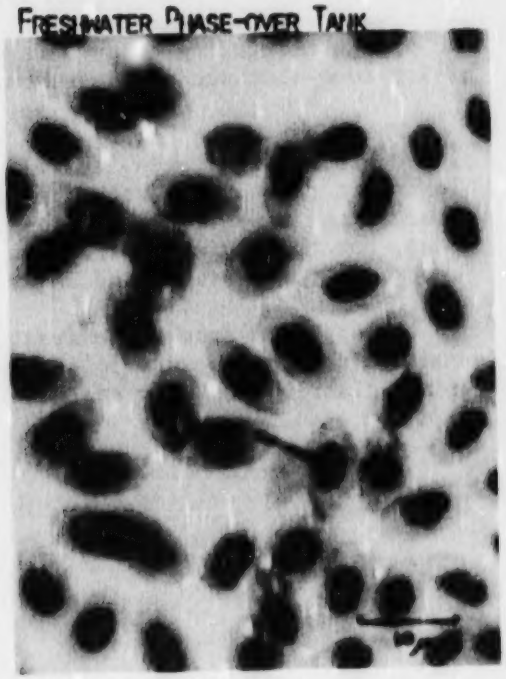


FRESHWATER - 4 HOURS

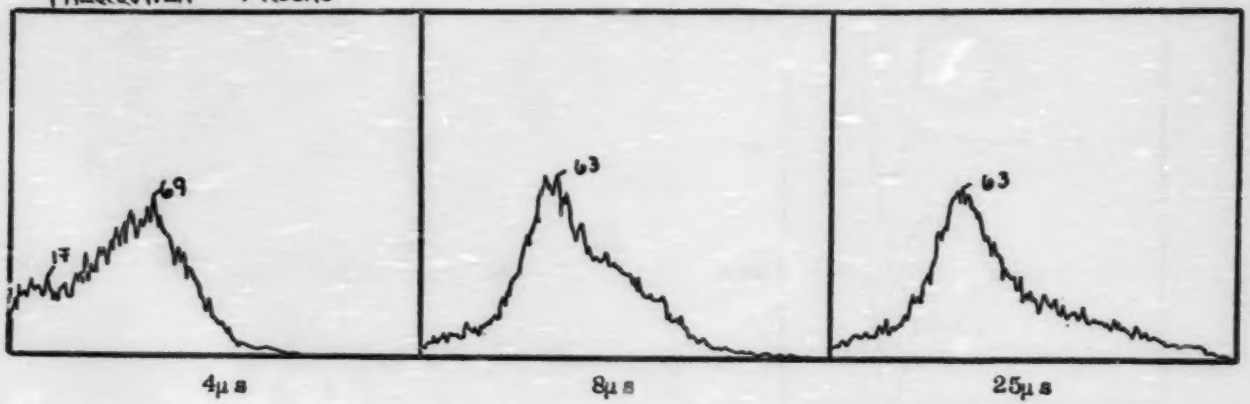
FRESHWATER - 6 HOURS



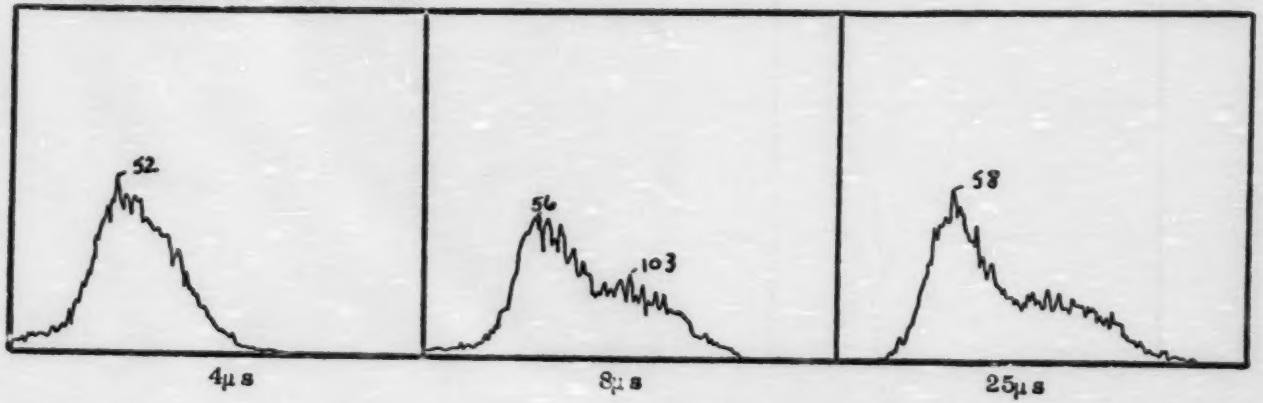
FRESHWATER PHASE-OVER TANK



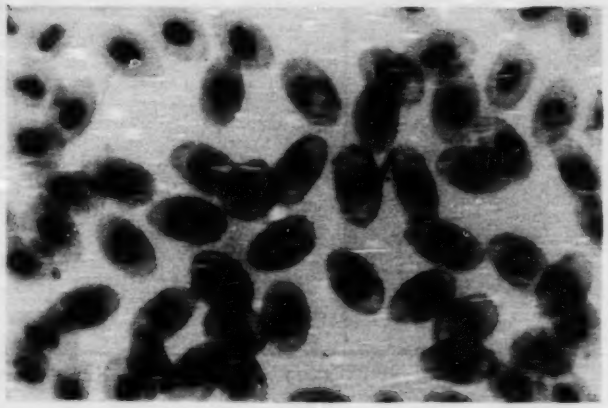
FRESHWATER - 4 HOURS



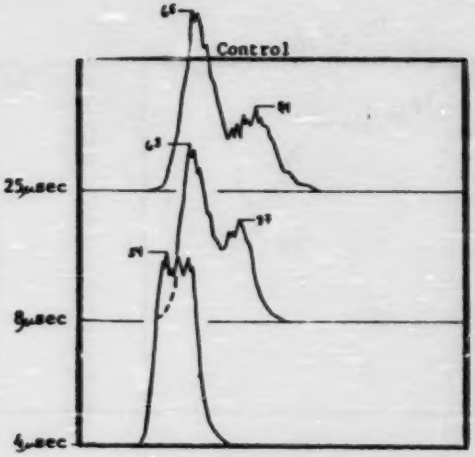
FRESHWATER PHASE-OVER TANK

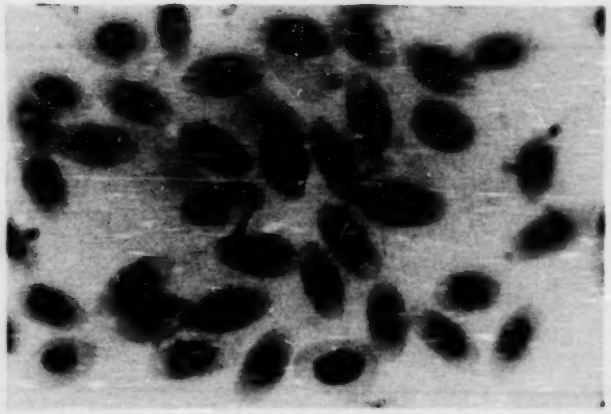


CONTROL VS. FRESHWATER ADAPTED

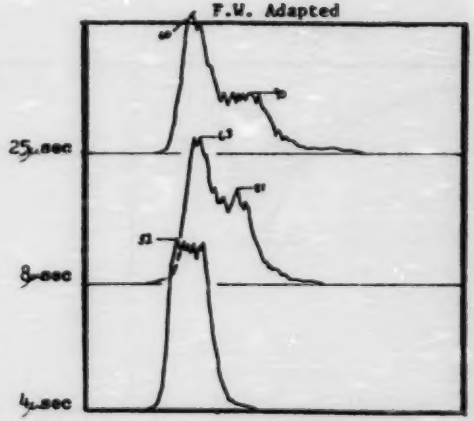


CONTROL

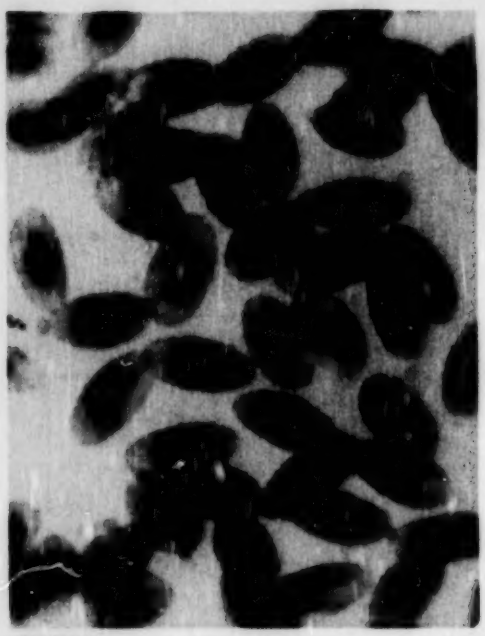




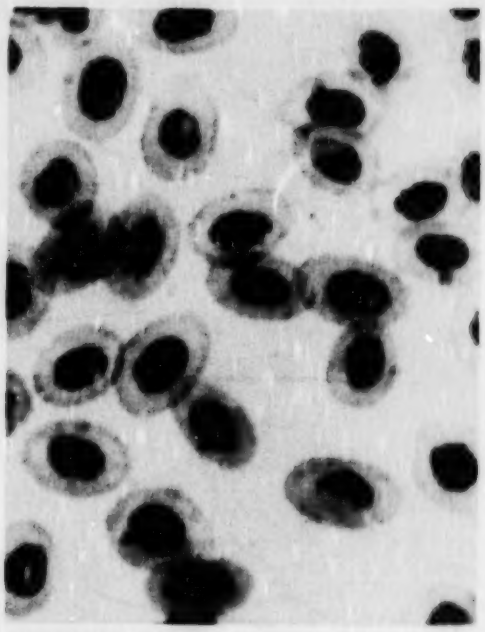
FRESHWATER ADAPTED



DISTILLED WATER vs. $CuCl_2$/DISTILLED WATER

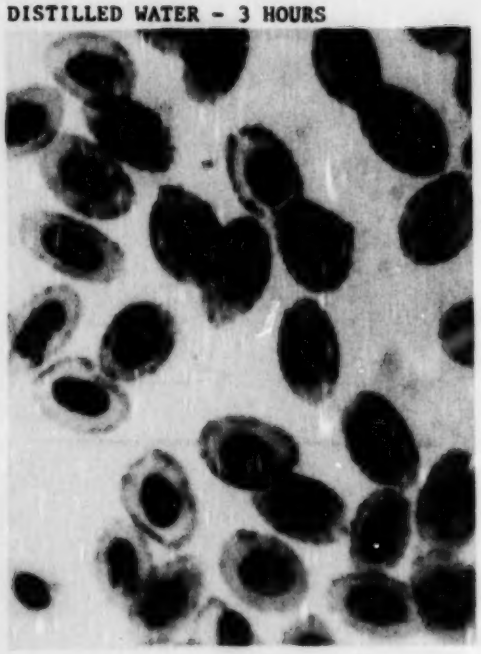


CONTROL

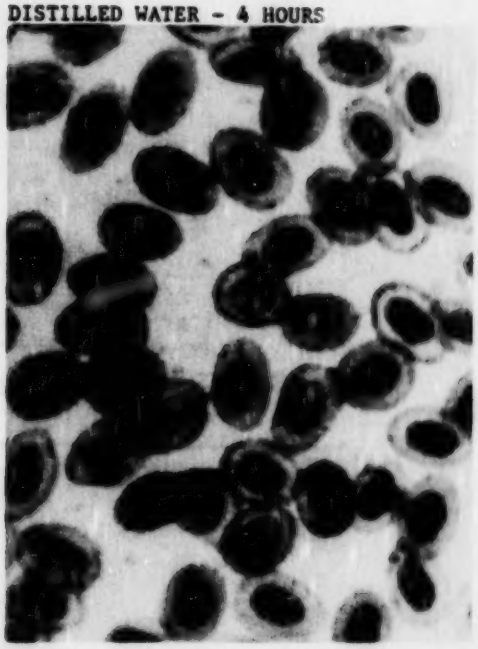


DISTILLED WATER - 1 HOUR

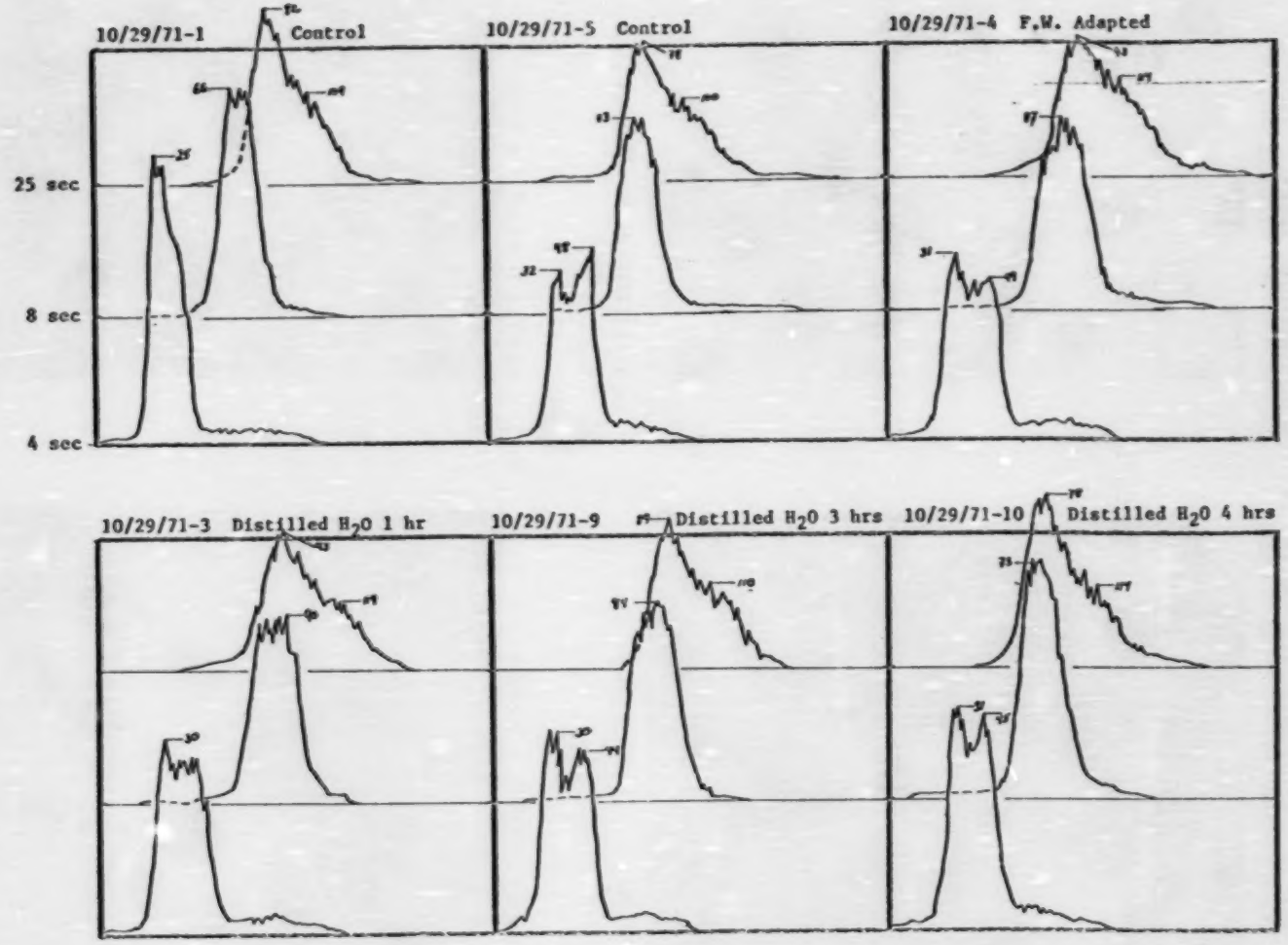
DISTILLED WATER - 3 HOURS



DISTILLED WATER - 4 HOURS



DISTILLED WATER



RESULTS

Cadmium Experiments

The primary experiment involving cadmium was a test done in collaboration with the EPA headquarters in West Kingston, Rhode Island. At the EPA facilities, Fundulus heteroclitus was exposed to 1ppm Cd^{++} for 10 months. After the 10 months, the fish were transported to our laboratory to be evaluated. Some of the fish were evaluated directly from the water they were transported in and some of the fish were placed in unpolluted saltwater for various intervals from 20.75 hours to 42 hours. The blood of these fish was examined on the BMA. This results of this chronic exposure test and the subsequent return of a small number of the fish to unpolluted saltwater are summarized in Tables I and II.

A short-term exposure to $CdCl_2$ was conducted at our laboratory. There was a problem in this experiment and the validity of the results are questionable. However, it served to point out some of the problems involved in determining the exact amount of a metal dissolved in either freshwater or saltwater. When the $CdCl_2$ was added to the freshwater experimental tank a certain amount precipitated out as $CdOH_2$. Thus, it was difficult to determine with any accuracy the amount of free Cd^{++}. The fish used in this cadmium experiment were fish taken from the freshwater adaptation tank. During the time of the test, the fish remained normal in both behavior and appearance. The saltwater controls and freshwater adapted fish were also normal in both behavior and appearance. Fish were sacrificed after 3.5 and 5.75 hours in the experimental tank.

Photomicrographs:

The dimensions of the cells and nuclei of the experimental fish in the short-term exposure were similar to the fish from their "parent" environment, the freshwater adapted tank (page 26).

It appeared from the statistical data as well as the appearance of the cells in the photomicrographs that there was little if any effect on the cells and the nuclei from the cadmium. Since most of the cadmium had precipitated out as $CdOH_2$, this non-effect would not be surprising.

Electronic Prinouts:

The printouts for the fish exposed to Cd^{++} for 3.5 and 5.75 hours in the three triggering delay intervals were fairly normal (page 27). In the 4μsec triggering delay interval, the single peak was slightly more spread than usual indicating more variance in cell widths than normally found. The 8μsec and 25μsec triggering delay intervals were normal in all cases; that is, a major primary peak followed by a smaller secondary peak with the 25μsec interval distribution broader than the 8μsec interval.

As noted in the photomicrographs, there did not seem to be any disruption in the normal cell state caused by the cadmium. The problem, as stated before, was determining how much Cd^{++} was available in solution.

TABLE I

FISH - IN CADMIUM AND REVERSED

Number	Metal ppm	Time Exposed	4 μsec Normal	4 μsec Cd	8 μsec Normal	8 μsec Cd	25 μsec Normal	25 μsec Cd	Observation
Control #23			X		X		X		
Cd-1	Cd 1 ppm	10 months	X			X		X	25 μsec No 2
Cd-2	Cd 1 ppm	10 months	X			X		X	
Control #26			X		X		X		
Cd-3	Cd 1 ppm	10 months		X		X		X	
Control #28			X		X		X		
Cd-4	Cd 1 ppm	10 months		X		X		X	
Cd-5	Cd 1 ppm	10 months		X		X		X	
Cd-6	Cd 1 ppm	10 months		X		X		X	
Cd-7	Cd 1 ppm	10 months		X		X		X	
Cd-8	Cd 1 ppm	10 months		X		X		X	
Cd-9	Cd 1 ppm	10 months		X		X		X	
Cd-10	Cd 1 ppm	10 months		X		X		X	
Control #30 PM			X		X		X		
Control #29 AM			X		X		X		
Cd-11	Cd 1 ppm	10 months	X			X		X	
Cd-12	Cd 1 ppm	10 months	X			X		X	
Cd-13	Cd 1 ppm	10 months	X			X		X	
Cd-14	Cd 1 ppm	10 months	X	X		X		X	
Cd-15	Cd 1 ppm	10 months		X		X		X	
Control #31			X		X		X		
Cd-R-1	Salt H_2O	20 3/4 hrs	X		X		X		
Cd-R-2	Salt H_2O	21 hrs	X		X		X		
Control #32			X		X		X		
Cd-R-3	Salt H_2O	41 3/4 hrs	X		X		X		
Cd-R-4	Salt H_2O	41.9 hrs	X		X		X		
Cd-R-5	Salt H_2O	42 hrs	X		X		X		

TABLE II

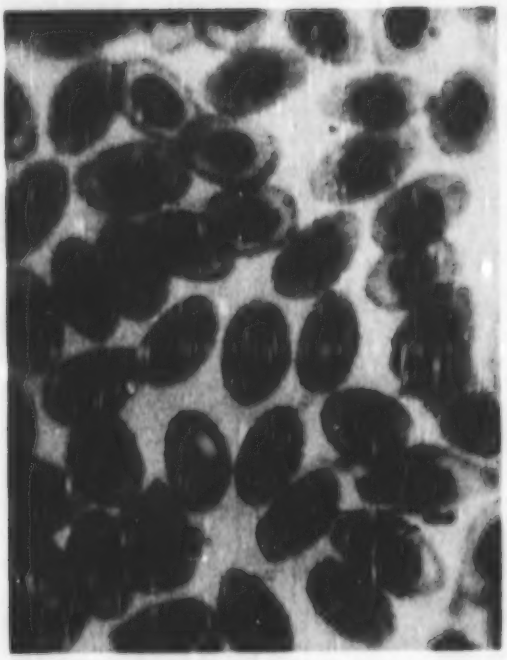
FISH - IN CADMIUM AND REVERSED

By Channel (Peak) Number

Number	Metal ppm	Time Exposed	4 μsec Normal	4 μsec Cd	8 μsec Normal	8 μsec Cd	25 μsec Normal	25 μsec Cd	Observation
Control #23			51		74		74,112		
Cd-1	Cd 1 ppm	10 months	52			88		93	25 μsec No 2
Cd-2	Cd 1 ppm	10 months	58			84		80,	
Control #26			58		88		81,		
Cd-3	Cd 1 ppm	10 months		65		96		89,	
Control #28			56		82		79,		
Cd-4	Cd 1 ppm	10 months		63		90		88	25 μsec No 2
Cd-5	Cd 1 ppm	10 months		65		104		98	" " "
Cd-6	Cd 1 ppm	10 months		60		94		92,	
Cd-7	Cd 1 ppm	10 months		71		103		97,	
Cd-8	Cd 1 ppm	10 months		65		103		93,	
Cd-9	Cd 1 ppm	10 months		75		115		93,	
Cd-10	Cd 1 ppm	10 months		61		91		83,	
Control #30 PM			50		83		79,		
Control #29 AM			54		80		77,		
Cd-11	Cd 1 ppm	10 months	53			91		91,	
Cd-12	Cd 1 ppm	10 months	52			92		90,	
Cd-13	Cd 1 ppm	10 months	54			103		108,	
Cd-14	Cd 1 ppm	10 months	58			109		110	25 μsec No 2
Cd-15	Cd 1 ppm	10 months		65		120		121	" " "
Control #31			41		78		79,		
Cd-R-1	Salt H_2O	20 3/4 hrs	53		85		81,		
Cd-R-2	Salt H_2O	21 hrs	50		74		70,		
Control #32			40		70		66,		
Cd-R-3	Salt H_2O	41 3/4 hrs	41		77		75,		
Cd-R-4	Salt H_2O	41.9 hrs	44		70		68,		
Cd-R-5	Salt H_2O	42 hrs	43		70		66,		

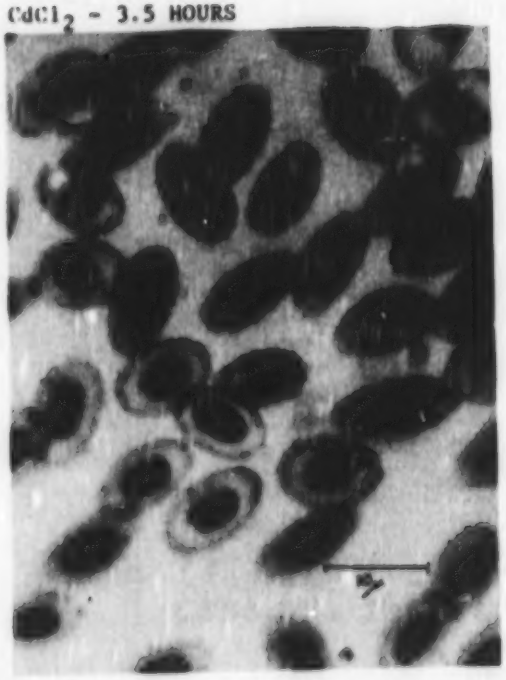
$CdCl_2$ in FRESHWATER

CONTROL

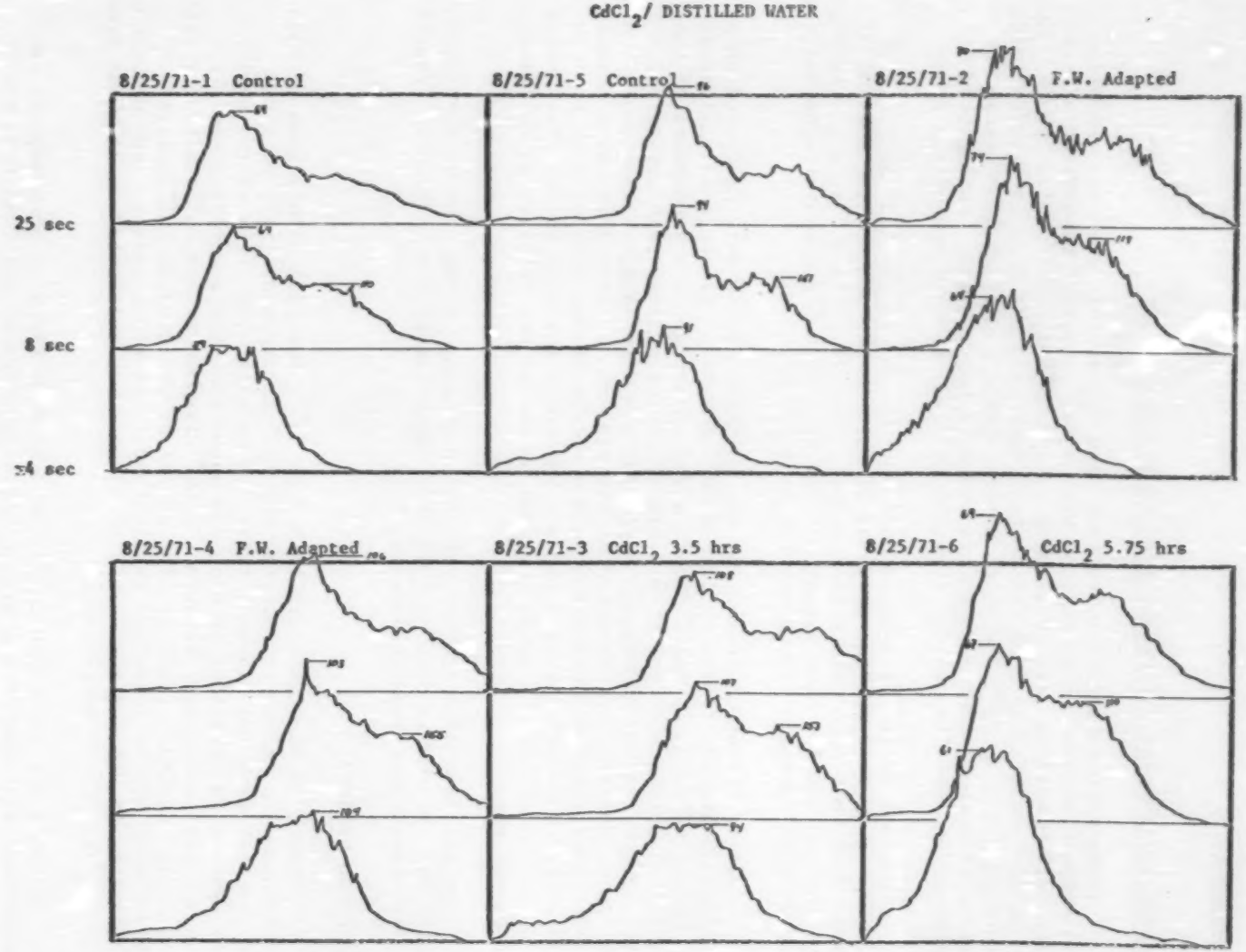


FRESHWATER-ADAPTED

$CdCl_2$ - 3.5 HOURS



$CdCl_2$ - 5.75 HOURS


$CdCl_2$/ DISTILLED WATER
8/25/71-1 Control
8/25/71-5 Control
8/25/71-2 F.W. Adapted
25 sec
8 sec
≥4 sec
8/25/71-4 F.W. Adapted
8/25/71-3 $CdCl_2$ 3.5 hrs
8/25/71-6 $CdCl_2$ 5.75 hrs

RESULTS

Copper Experiments - Freshwater vs. Saltwater

Tests were conducted to compare the ability of freshwater adapted and normal saltwater fish to cope with a Cu^{++} insult. Freshwater adapted fish were placed in jars containing 1.5ppm Cu^{++} diluted in freshwater. Saltwater fish were taken from the control tanks and put into jars of saltwater containing 1.5ppm Cu^{++}. Some fish were sacrificed after 24 hours in the test environment; others were left fro a 96 hour period. During this 96 hour period, one fish in Cu^{++}/freshwater died, another was nearly dead.

The fish in Cu^{++}/freshwater did not eat during the test period; not did they seem to sense the presense of food in the water. The eyes of these fish were much darker than normal. However, these fish did swim about the jar and did not seem sluggish. The fish in Cu^{++}/saltwater appeared and behaved normally throughout the test period.

Another series of freshwater vs. saltwater tests were conducted using a slightly higher concentration of Cu^{++} (2ppm). Fish were sacrificed at intervals of 1, 3, 5, and 24 hours after exposure. The appearance of the test fish during the first few hours of the experiment remained normal. The fish behaved fairly normally, though perhaps more sluggish than usual. After 24 hours, however, the behavior of the fish in the test jars was abnormal. They either remained motionless on the bottom of the jars or swam rapidly and erratically with jerky motions. When fed, the fish in Cu^{++}/freshwater did not eat or seem to sense the food in the water; the fish in Cu^{++}/saltwater did eat.

At the time of sacrifice, the blood of some of the fish in Cu^{++}/freshwater was abnormally dark and viscous. Only one of the fish in Cu^{++}/saltwater showed this same phenomenon.

Photomicrographs:

After a 24 hour exposure to 1.5ppm Cu^{++}, the cell size values in both freshwater and saltwater experimental fish corresponded well to those of the fish from the "parent" tanks. Generally, the cells of the test fish were more irregular in size and shape than the controls (page 31). The greatest difference between the Cu^{++}/freshwater and Cu^{++}/saltwater fish was in the nuclear staining. The nuclei of the fish in Cu^{++}/saltwater showed what may be described as light-spot staining; that is less dense spots or streaks in the nucleus. This phenomenon was consistent in all fish from the Cu^{++}/saltwater environment.

At 96 hours in 1.5ppm Cu^{++}, the cell dimensions of both sets of experimental fish were still within the size range of the fish from the "parent" tanks. However, there was a definite change in the nuclei of the experimental fish. The lengths of the nuclei in both sets of fish (freshwater and saltwater) were longer than the controls (page 33). The nuclei of the fish in Cu++/saltwater still exhibited light-spot staining. The cell

shapes of these fish as well as the Cu^{++}/freshwater fish were also irregular. One of these fish, which was nearly dead at the time of sacrifice, had cells and nuclei which were greatly disrupted; this disruption consisted of swelling and bursting cells, a phenomenon seen often in dying fish.

The data from the photomicrographs seem to indicate that whatever happens on a cell membrane or cytoplasmic level, at this copper concentration, happens within a few hours after the exposure to copper. After 24 hours, it appears that the nucleus is the focus of change.

In the previous experiments, the effects of exposure to copper for a few hours was not tested. Since some important membrane phenomena may have been missed, tests were conducted to determine the more immediate effects of 2ppm Cu^{++} of the fish cells. Comparing the experimental fish with the fish from the "parent" tanks, a number of striking differences were found. While the cell widths of both sets of experimental fish remained the same as the controls, the cell length changed strikingly (35, 36). The situation of cells swelling, which has often been seen, did not occur here; the cells changed in length only. Thus, another process seemed to be taking place. The cells of the Cu^{++}/freshwater fish were shorter and rounder than normal, and the cells of the Cu^{++}/saltwater fish were more elongated. Further, the changes did not appear to increase with time elapsed. The greatest change seemed to occur between the first and second hours.

After 24 hours in a 2ppm Cu^{++} environment, the size values were within a range of values shown by the fish from the "parent" tanks. The difference was that the variance had increased. The cell length was again the most affected dimension; the cell lengths of the fish in Cu^{++}/freshwater were still relatively small; and those of the fish in Cu^{++}/saltwater had decreased considerably and come into line with the controls (page 38).

It was noted in the introduction to this section that several fish were observed to have dark, viscous blood at the time of sacrifice. These fish had blood cells that were extremely short and rounded. These observations appear to indicate that there is possible interference with the oxygenation of the blood. This phenomenon seemed to affect the fish in the Cu^{++}/freshwater environment to a greater extent than the fish in Cu^{++}/saltwater.

Electronic Printouts:

In discussing the mensurational data from the photomicrographs, it was noted that the cell sizes did not vary greatly from the values of the controls. In analyzing the printouts, it will be remembered that it is the cell width which is the critical dimension.

In the case of the exposure to 1.5ppm Cu^{++} for 24 and 96 hours, it was the cell nuclei which were the focus of change. Although the cells were less regular in size and shape than the controls, the cell size values were within the limits of the controls. The electronic printouts reflected this situation (32, 34). While the peak channel does not change greatly

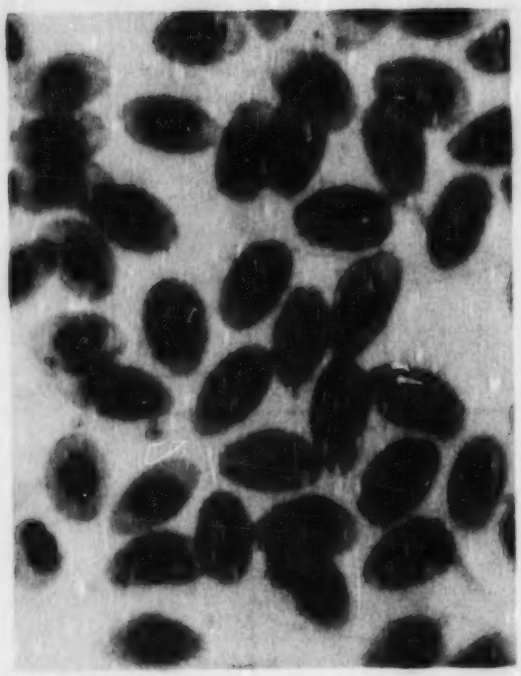
the distributions do differ from the normal, indicating changes in shape not size.

In an exposure of 1 hour to 2ppm Cu^{++} , the cell length was the affected dimension. But since the electronic printouts are a reflection of cell width, and this dimension did not change, the printouts did not show an abnormal size distribution. Fish subjected to 2ppm Cu^{++} for 3 and 5 hours did show abnormal electronic assays (page 37).

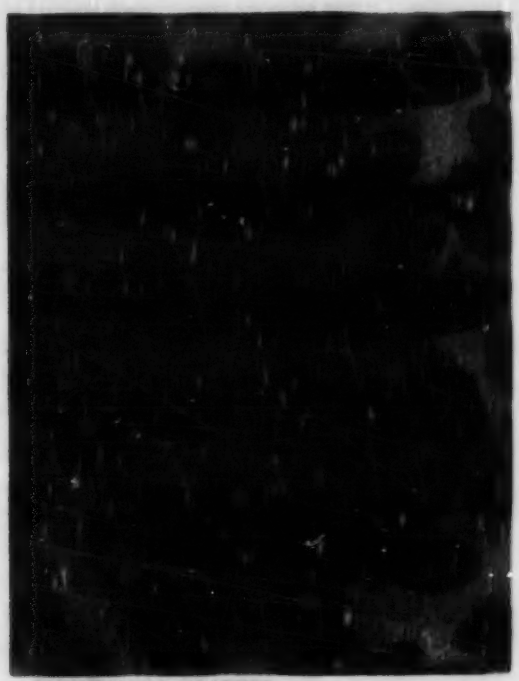
All of these electronic distributions must be studied on terms of the relationships of the triggering delay intervals to one another. In a normal cell state, the major peaks of the three triggering delay intervals fall almost in the same channel, this indicates similarity of size within a cell population. But in a changed cell state, as the secondary peak of the 8μsec triggering delay interval begins to disappear, the peak of the 4μsec triggering delay interval shifts away from the main peak of the 8μsec triggering delay interval. The 25μsec triggering delay interval follows the same pattern as the 8μsec interval.

In the freshwater vs. saltwater experiments, the longer the fish were exposed to Cu^{++} the greater the number of fish which had separated peaks in the electronic printouts. The same held true the greater the concentration of Cu^{++} which was used. In addition, when comparing the Cu^{++}/freshwater and Cu^{++}/saltwater environments, the fish in the Cu^{++}/freshwater were the first to show secondary peak reduction and peak separation. It appeared that the freshwater adapted fish when subjected to further environmental stress were more vulnerable to the added insult.

$CuCl_2$/SALTWATER vs. $CuCl_2$/FRESHWATER

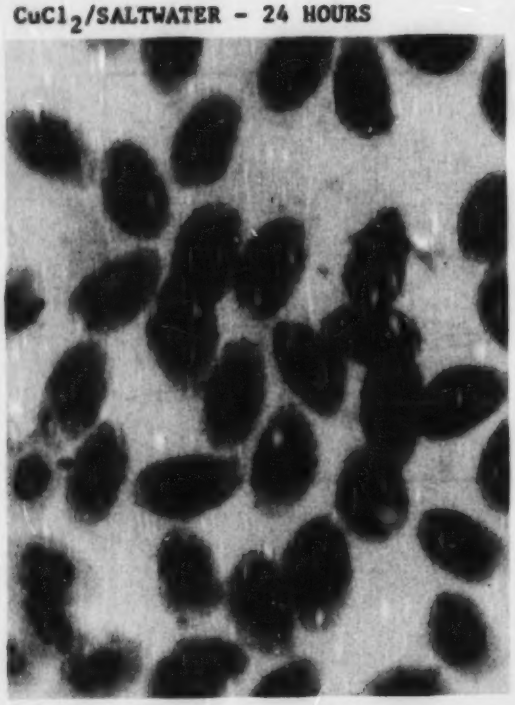


CONTROL

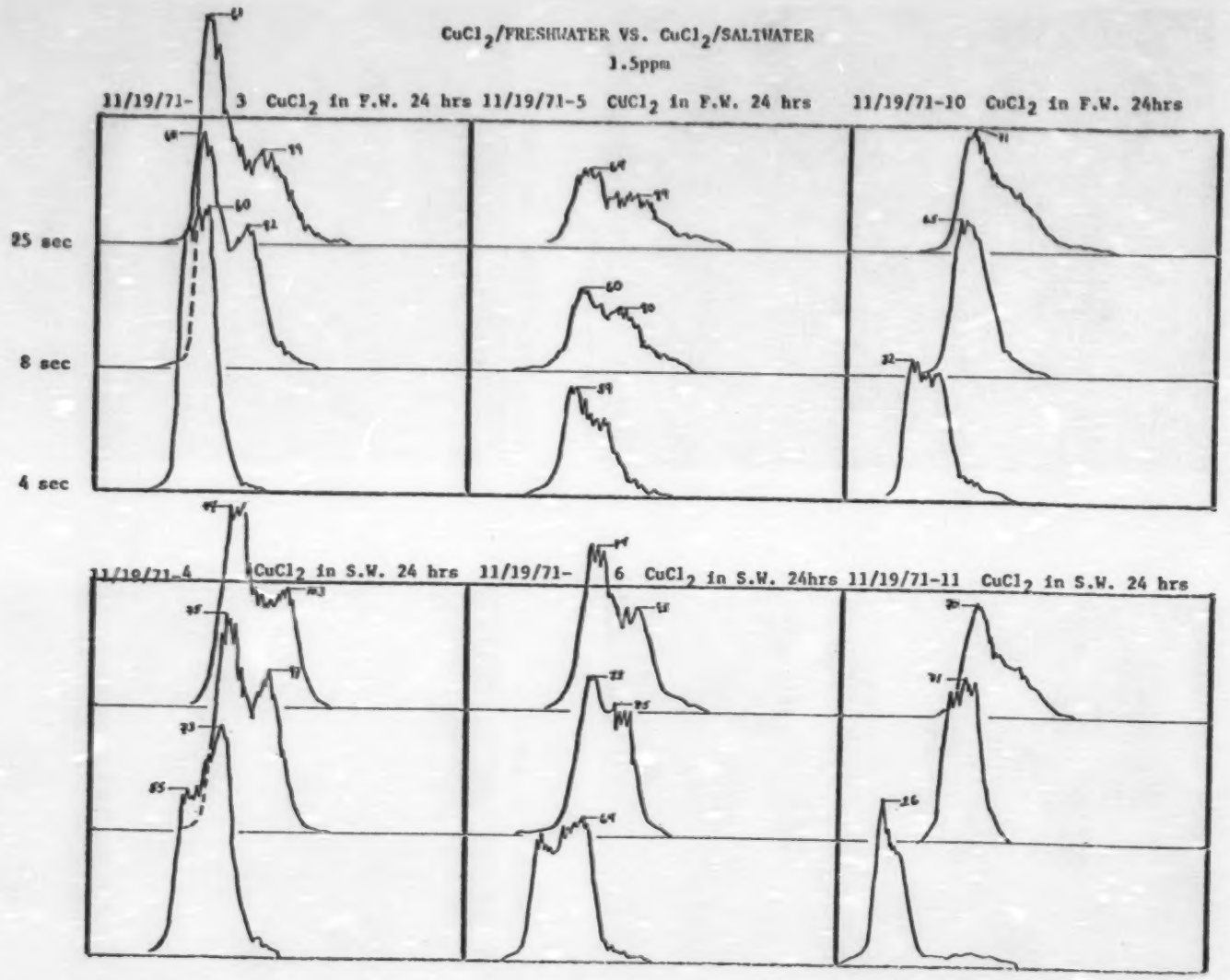


FRESHWATER-ADAPTED

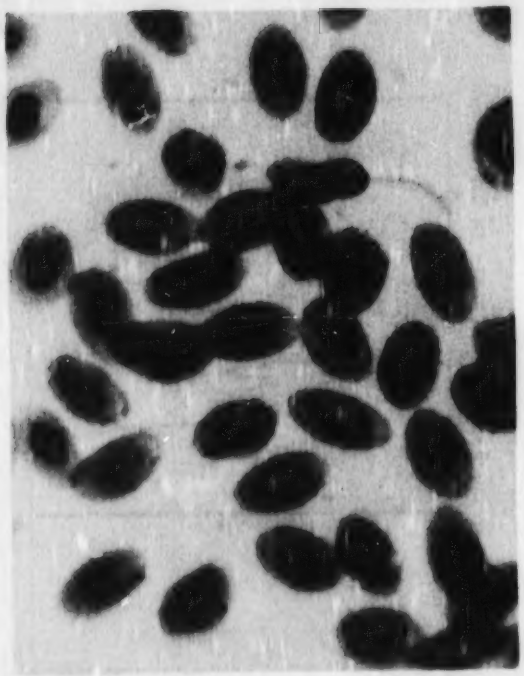
$CuCl_2$/SALTWATER - 24 HOURS



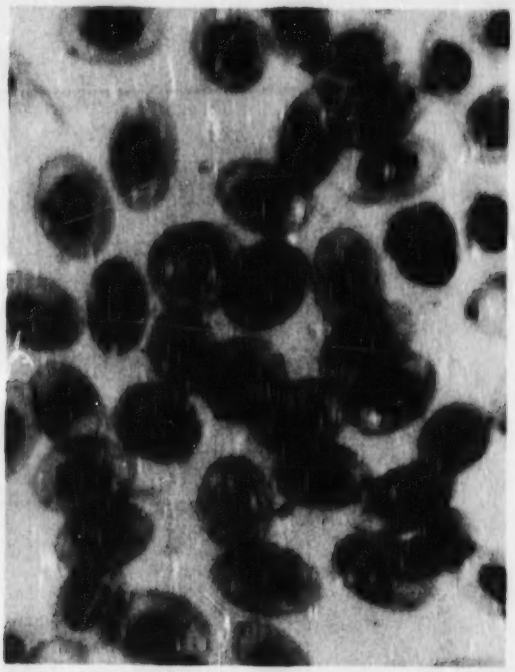
$CuCl_2$/FRESHWATER - 24 HOURS


$CuCl_2$/FRESHWATER VS. $CuCl_2$/SALTWATER
1.5ppm
11/19/71- 3 $CuCl_2$ in F.W. 24 hrs
11/19/71-5 $CuCl_2$ in F.W. 24 hrs
11/19/71-10 $CuCl_2$ in F.W. 24hrs
25 sec
8 sec
4 sec
11/19/71-4 $CuCl_2$ in S.W. 24 hrs
11/19/71- 6 $CuCl_2$ in S.W. 24hrs
11/19/71-11 $CuCl_2$ in S.W. 24 hrs

$CuCl_2$/SALTWATER vs. $CuCl_2$/FRESHWATER

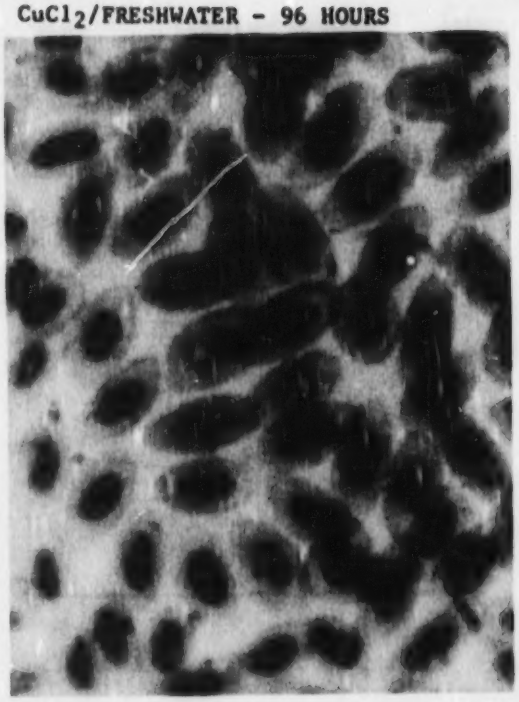


CONTROL

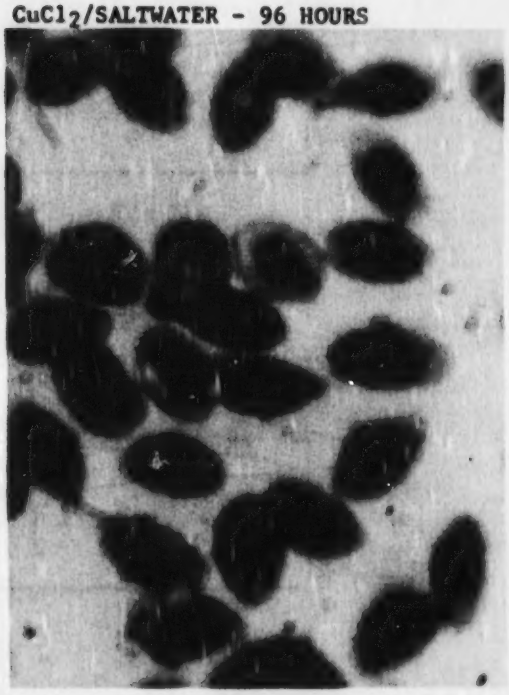


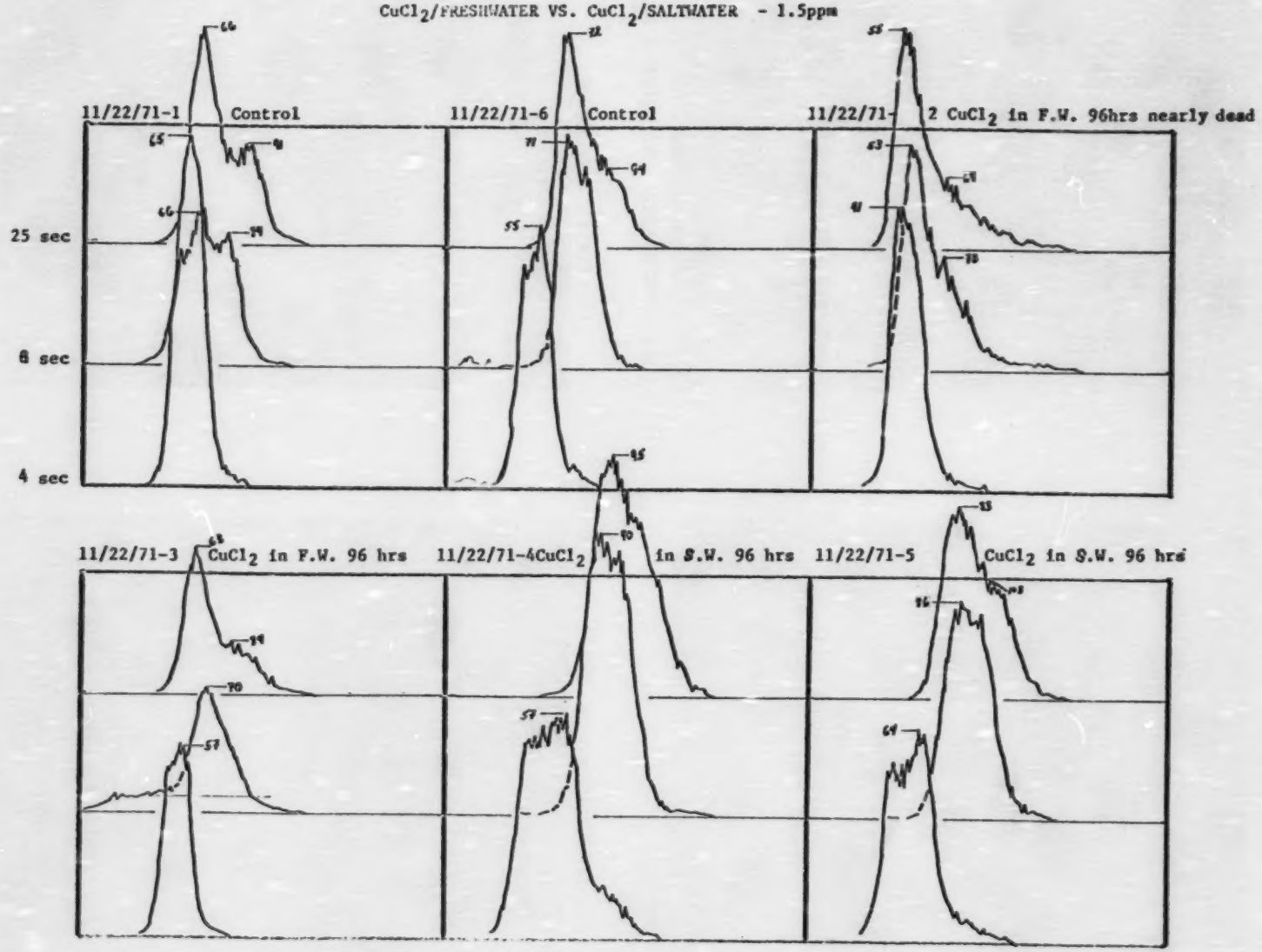
$CuCl_2$/FRESHWATER - 96 HOURS - NEARLY DEAD

$CuCl_2$/FRESHWATER - 96 HOURS

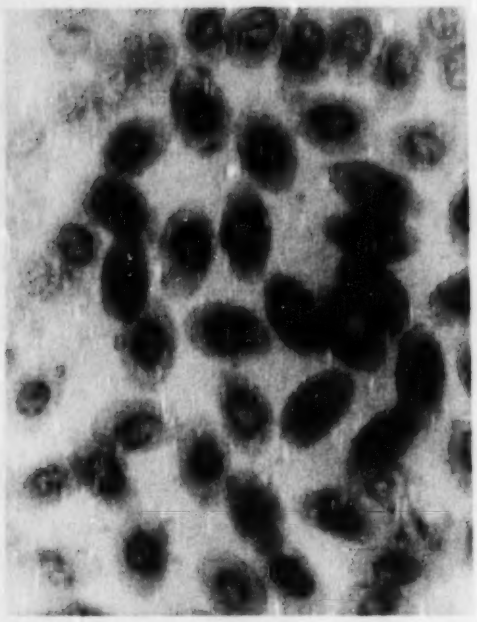


$CuCl_2$/SALTWATER - 96 HOURS

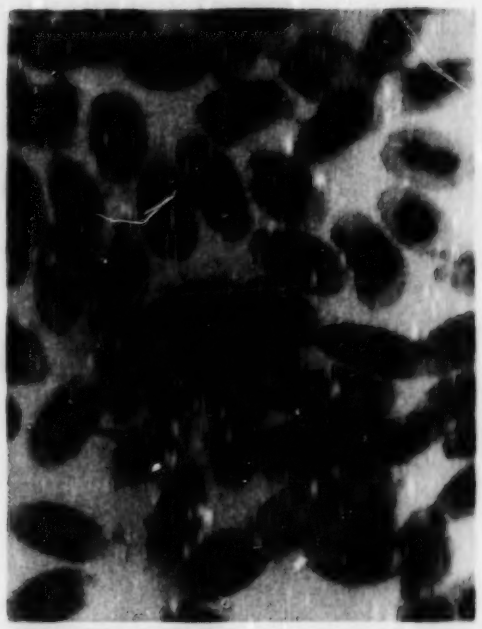



CuCl2/FRESHWATER VS. CuCl2/SALTWATER - 1.5ppm
11/22/71-1 Control
11/22/71-6 Control
11/22/71- 2 CuCl2 in F.W. 96hrs nearly dead
25 sec
8 sec
4 sec
11/22/71-3 CuCl2 in F.W. 96 hrs
11/22/71-4CuCl2 in S.W. 96 hrs
11/22/71-5 CuCl2 in S.W. 96 hrs

$CuCl_2$/SALTWATER vs. $CuCl_2$/FRESHWATER

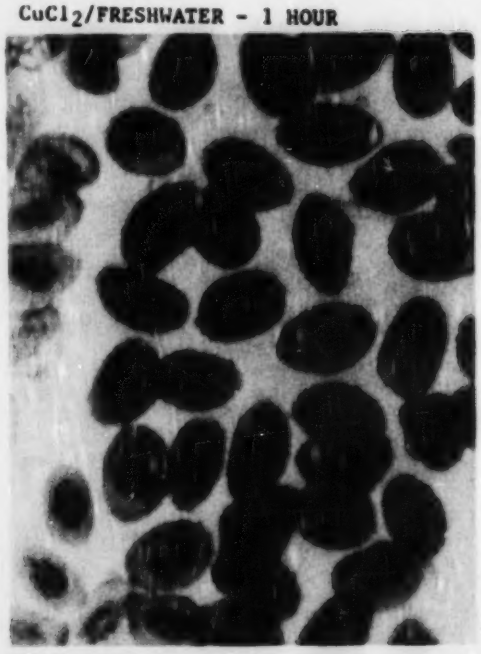


CONTROL

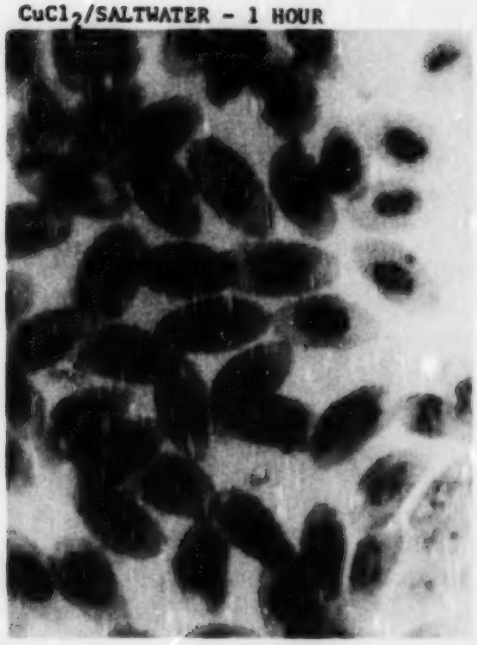


FRESHWATER-ADAPTED

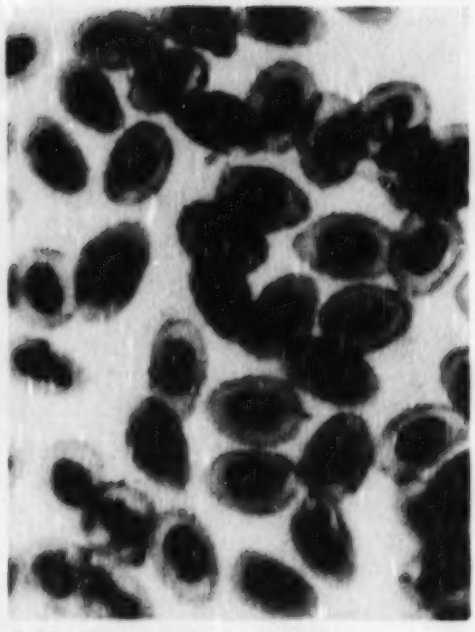
$CuCl_2$/FRESHWATER - 1 HOUR



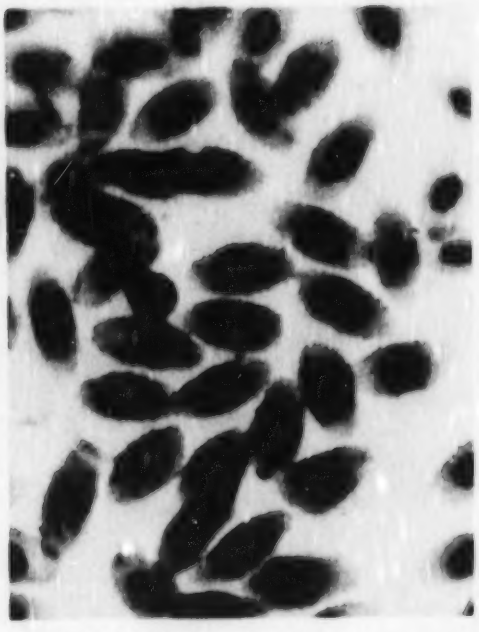
$CuCl_2$/SALTWATER - 1 HOUR



$CuCl_2$/SALTWATER vs. $CuCl_2$/FRESHWATER

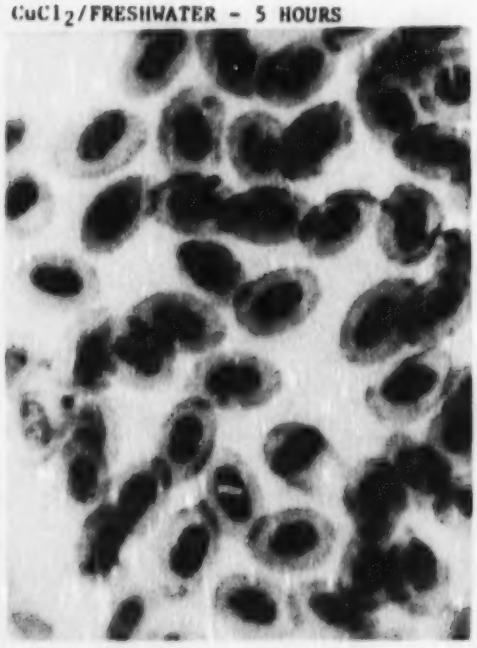


$CuCl_2$/FRESHWATER - 3 HOURS

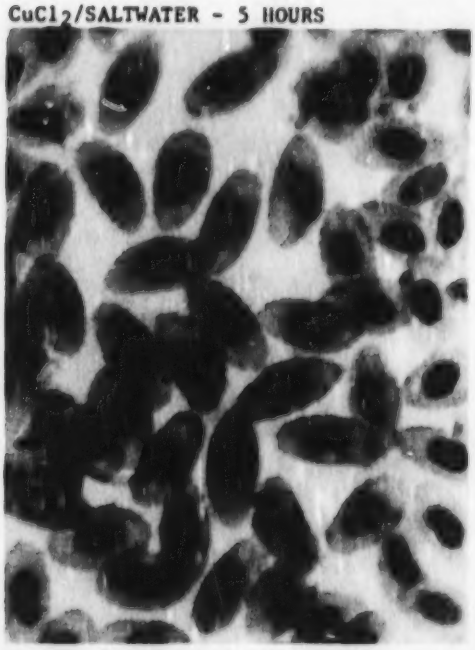


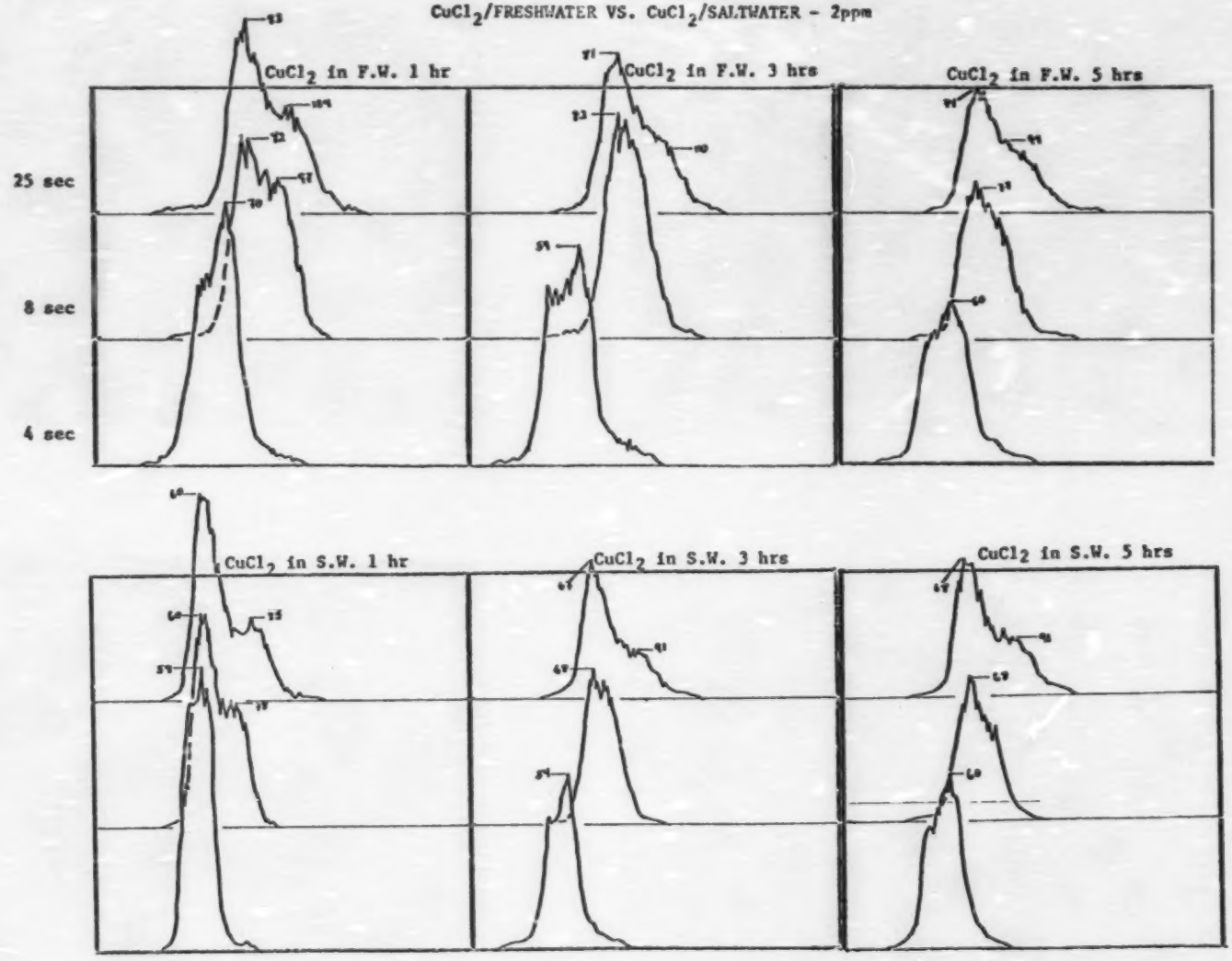
$CuCl_2$/SALTWATER - 3 HOURS

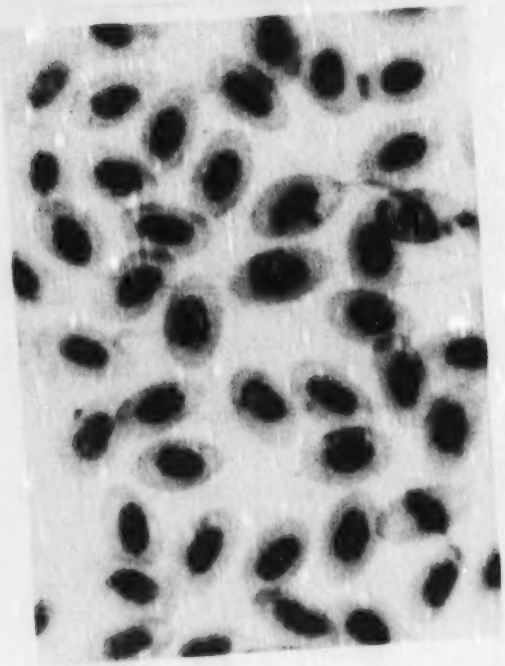
$CuCl_2$/FRESHWATER - 5 HOURS



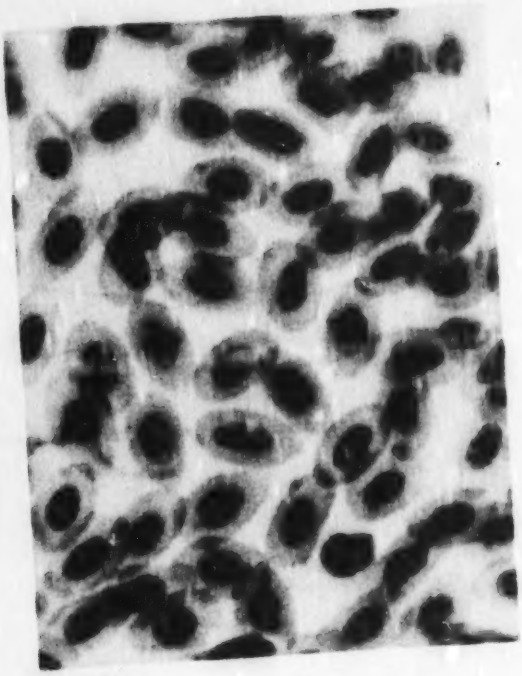
$CuCl_2$/SALTWATER - 5 HOURS



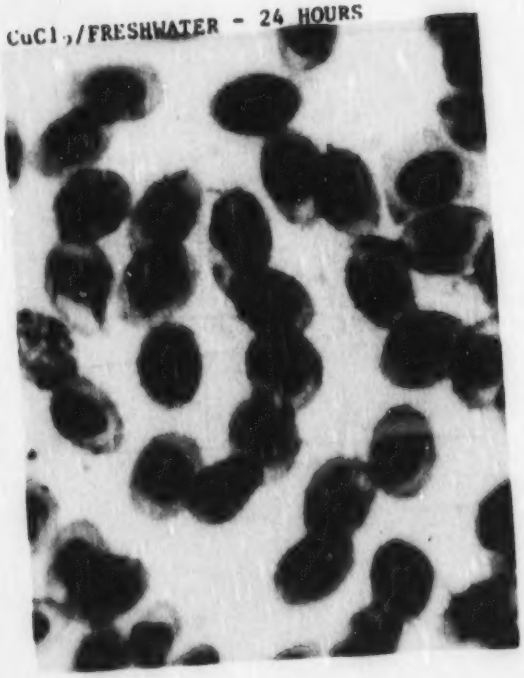

CuCl2/FRESHWATER VS. CuCl2/SALTWATER - 2ppm
CuCl2 in F.W. 1 hr
CuCl2 in F.W. 3 hrs
CuCl2 in F.W. 5 hrs
25 sec
8 sec
4 sec
CuCl2 in S.W. 1 hr
CuCl2 in S.W. 3 hrs
CuCl2 in S.W. 5 hrs



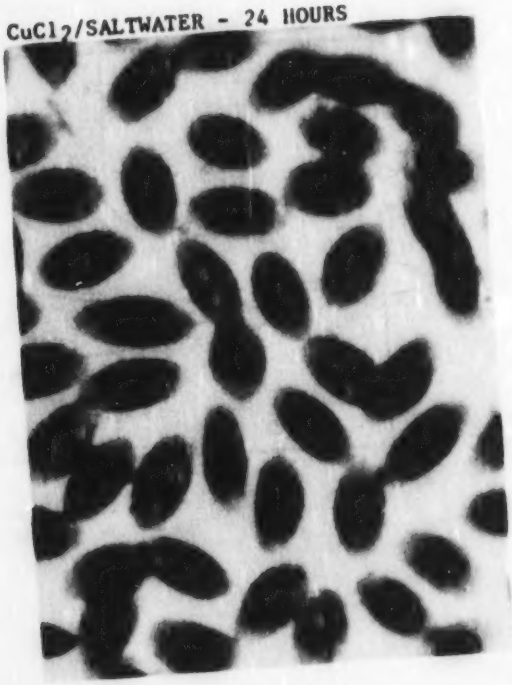
CONTROL



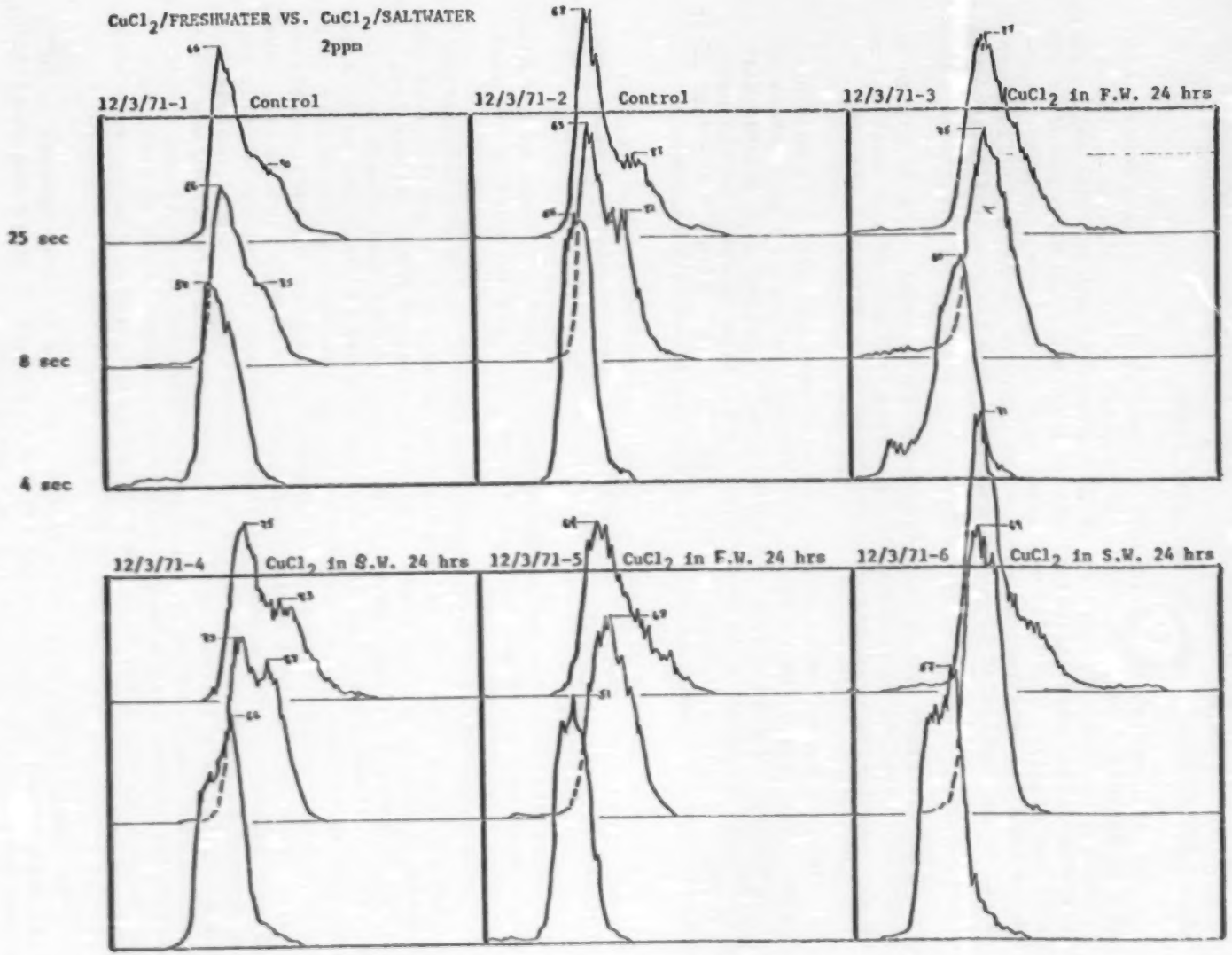
CONTROL



$CuCl_2$/FRESHWATER - 24 HOURS



$CuCl_2$/SALTWATER - 24 HOURS


$CuCl_2$/FRESHWATER VS. $CuCl_2$/SALTWATER
2ppm
12/3/71-1 Control
12/3/71-2 Control
12/3/71-3 $CuCl_2$ in F.W. 24 hrs
25 sec
8 sec
4 sec
12/3/71-4 $CuCl_2$ in S.W. 24 hrs
12/3/71-5 $CuCl_2$ in F.W. 24 hrs
12/3/71-6 $CuCl_2$ in S.W. 24 hrs

RESULTS

Copper Experiments - 5ppm and Recovery Tests

For this series of experiments, a concentration of 5ppm copper was used to provoke a quick and dramatic response to the pollutant. The experimental protocol was similar to that described before. One gallon glass jars were filled with saltwater, the experimental fish were placed in the jars, and finally, the $CuCl_2$ was added to the jars. The recovery experiments used the same test set-up. After exposure to Cu^{++}, the fish were placed in an unpolluted 15-gallon saltwater tank for a specified recovery period.

Exposure times varied from 45 minutes to 44 hours. The 45 minute exposure was designed to characterize the early effects of the metal on the cells. Short-term exposures were continued for 4.5 and 5 hours.

The progression of exposure times was continued with a 24 hour exposure. The results up to this point did not indicate a clear series of of reactions. The 45 minute exposure to Cu^{++} resulted in a greater disruption of the normal cell state than the 4.5 hour exposure to the metal. This may indicate a dramatic initial reaction which looses its effect after a time; that is a reaction only involving the blood cell membrane.

We wished to determine whether this immediate effect was the only effect. Therefore, the time exposure to Cu^{++} was increased to determine whether the reaction to Cu^{++} was only transient.

After 24 hours, the cells were greatly disrupted. To estimate whether this damage would progress further, the exposure was extended to 44 hours.

Short-term reversal experiments were carried out to get information on the ability of the fish to recover from an insult by a metal. A 24 hour exposure to the metal was chosen, as we had observed this time was long enough to cause definite damage to the cells, but not long enough to kill the fish. It was found that exposure for 24 hours to $CuCl_2$ caused internal not just superficial injury to the cells. We wished to learn whether this kind of injury permanently or only temporarily altered the affected blood cells. Fish in a natural environment exposed to chronic, not lethal, levels of a pollutant may seem normal in behavior and appearance, but in fact may be damaged in a way that interfers with reproductive or social behavior or their ability to withstand any further environmental stress. It was hoped that the reversal experiments would provide information on 1) the fish's ability to recover from a heavy metal insult, 2) the level of metal in the water with which the fish are unable to cope, and 3) the exposure time in the polluted water after which the fish cannot recover.

Two recovery intervals were tested. After a 24 hour exposure to Cu^{++}, fish were placed in the saltwater recovery tank for 10 hours and sacrificed; others were left for one week and then sacrificed.

Photomicrographs:

After a 45 minute exposure, both phase contrast studies from fresh blood and bright-field studies of the Wright stained cells showed many irregularities in cell shapes.

In phase contrast microscopy, some cells appear in a "u" or "v" formation, others showed a saucer effect or were in other abnormal shapes (page 46). Many still had the smooth edge on the plasma membrane, others showed a jagged sword effect. The nuclei appeared normal, but as they had not been satined no details could be seen. An uneven cytoplasmic transparency was observed which was not present in the control erythrocytes, and at the same time some cells had an opaque appearance.

In the photomicrographs, many of the Wright stained appeared abnormal in shape (page 46). The cytoplasmic membrane had lost its rigor and a number of odd-shaped cells were evident. Although about half the cells have retained the typical oval shape, many of the cells were swollen and rounded, some had a sickle shape. The cytoplasm was much more transparent than in the controls, and some showed an uneven clearing as in the phase contrast studies. The nuclei exhibited the same granular mottled appearance seen in the control cells. The nuclei of these cells were slightly more rounded than the controls. Some of the nuclei were off-center. A few of the nuclei were lobed.

In this 45 minute exposure to Cu^{++}, the greatest disturbance seemed to be to the cytoplasmic membrane. The effects of the metal ranged from a distortion of the cell shape to a total breakdown of the cytoplasmic membrane. The nuclei seemed little affected by the metal.

Most of the cells exposed to Cu^{++} for 4.5 to 5 hours maintained their cytoplasmic membrane intact. Most of the cells have retained an oval shape, though some were quite swollen (page 47). Some of the cells exhibited protrusions of the cytoplasm similar to pseudopodia. In a few of the cells, the cytoplasm appeared to be breaking down. Most of the nuclei seemed generally unaffected. A few of the nuclei were more round than oval, and some were not centrally located. The stain was taken up uniformly, except in the swollen cells in which the nuclei were lighter in color.

After 24 hours, the phase contrast studies of the erythrocytes from fresh blood showed definite atypical characteristics. Some had long points or spear shapes, others had clear white holes resembling vacuoles in the cytoplasm in a characteristic pattern (page 49). In many cells, the plasma membrane did not stand out as in the phase contrast control cells. Some of the cells had sickle shapes clearly outlined with the phase optics. The nuclei were of the normal oval shape. With phase contrast studies, white clearings were visible in some cell nuclei disclosing mottling of the chromatin, while others exhibited full clearing of the nucleus.

It can be seen in the photomicrographs of the Wright stained cells that after 24 hours in Cu^{++}, the cytoplasmic membrane had lost its rigor.

The edges were jagged and irregular (page 49). In many cases, the cytoplasm barely surrounded the nucleus. The cytoplasm of many cells seemed to have a cohesive property causing some of the cells to become aggregated. Although the cytoplasmic membrane appeared to have lost its rigidity and the cytoplasm appeared to be breaking down, there were few free nuclei. The nuclei varied greatly in size and shape. In some of the photomicrographs (not included) the cells and nucleihad swollen enormously. The nuclei had stained unevenly; the erythrocytes showed large variations in the transparency of the cytoplasm. It would seem that the effects at this 24 hour exposure went beyond the transient effects to the membrane, since the nuclei were also affected by the copper.

After a 44 hour exposure to $CuCl_2$, the same clearly defined pattern of deleterious effects is observed from the phase contrast photomicrographs. A sickle cell phenomenon was visible with the cells having a smooth plasma membrane (page 50). White clearings were consistently observed in the cytoplasm usually in two's with one clear spot or vacuole at each end of the cell. Some irregularily shaped cells differing from the above were present, a few with jagged plasma membranes. Mixed in with the other cells were normal appearing cells. In some cases, the nuclei were more transparent than those observed in the controls. The mottled chromatin was visible.

The Wright stained cells in the photomicrographs showed a greater disturbance of the normal state of the blood cells than the 24 hour exposure to $CuCl_2$ (page 50). The cytoplasmic membrane again was seen to have lost its rigor. The cytoplasm protruded, similar to pseudopodia, in certain instances. In other cells, the cytoplasm appeared to be breaking down and freeing the nucleus. The cytoplasm of some of the cells seemed to have meshed together. Several cells resembling thrombocytes were seen in the photomicrographs (page 50). The nuclei were stained unevenly, some nuclei were swollen and rounded. The most swollen nuclei were those whose cytoplasm had begun to break down.

In the first reversal experiment, fish were exposed to $CuCl_2$ for 24 hours and then returned to unpolluted saltwater for 10 hours. The phase contrast photomicrographs showed theerythrocytes to have a similar morphological structure to those observed after 24 hours in $CuCl_2$, i.e. irregular shapes, plasma membrane indentations, atypical edges, and a sickling phenomenon. The return for 10 hours to the normal slatwater environment did not restore the red blood cell population to that seen in a normal control (page 52). A typical thrombocytic network was seen in fresh blood preparations in phase contrast photomicrographs (page 52). Pseudopods of thrombocytes linked with the pseudopods of other thrombocytes. Erythrocytes that came into contact with the thrombocytic network became enmeshed.

The photomicrographs of the Wright stained cells showed very swollen, rounded cells. There were very few of the normal oval shaped cells. The cytoplasmic membrane, though not normal, seemed to have regained some rigor (page 52). Numerous cells still exhibited the cytoplasmic protrusions and jagged irregular edges, suggesting an injured plasma membrane unable to

return to its normal shape. The cytoplasm of many of the cells was quite transparent. The nuclei were also much more rounded than oval. Some were quite swollen and these did not take up the stain well; they were much lighter in color. All the nuclei had the granular appearance seen in the normal nuclei. The chief difference between these cells and those seen after an exposure of 44 hours was that there were fewer free nuclei and the cell cytoplasm, though extremely swollen, at least had remained intact. The fact that there was some rigor to the membrane may indicate a step towards recovery, though the cells seen in the photomicrographs were far from normal. The cytoplasm and the nuclei were still affected by the copper insult. The numerous cells with a frayed and irregular cytoplasmic membrane also indicated the inability to return to a normal condition in this 10 hour recovery period.

The return to unpolluted saltwater after a 24 hour exposure to $CuCl_2$ was continued for one week. Although some normal survivor erythrocytes were present, as in all studies, nevertheless, phase contrast photomicrographs revealed cells which continued to show injury. The beginning of a recovery was evident in the disappearance of the sickled shapes, irregular edges and plasma membrane injuries observed in the cells studied after 24 hours in a $CuCl_2$ environment without restoration to normal saltwater (page 53). However, most of the cells lacked the true normal control oval shape. Signs of persistent internal injury were evident in most of the cells. Irregular white clearings or vacuolations appeared in the cytoplasm of most of the cells. Irregular sword-shaped plasma membranes similar to those seen in the membranes of erythrocytes after one hour in a copper environment were still present in some cells. This might suggest an attempt at plasma membrane restoration. The nuclei of many cells instead of revealing the dark opaque effect of the normal control cells were transparent. This may indicate a clearing of the chromatin clumps and suggest a permanent copper involvement with the nuclear material. In the phase contrast photomicrographs, erythrocytes containing clear cytoplasm and some cytoplasmic inclusions were present. The thrombocytic network was seen again.

The high-dry photomicrograph (page 53) was of particular interest in that it provided an overall idea of the RBC population. This photomicrograph depicted a very mixed population of both immature, mature and nonviable erythrocytes. This was an unusual situation in that normally there were few immature cells in the circulating blood (Ref. 11).

Some of the Wright stained cells of these fish seemed to show signs of recovery. The cytoplasmic membrane shapes of these cells, though still atypical, appeared to have approached the uniform shape of the controls (page 53). The membranes appeared to be faint and jagged at the edges. In general, the cells resembled those exposed to $CuCl_2$ for 45 minutes. The nuclei were more oval than previously seen. Most of the nuclei were centrally located, another indication of a return of the red blood cell population towards normalcy. There were few free nuclei. The Wright stain seemed to have been taken up uniformly by the cell nuclei. After a 24 hour exposure to 5ppm $CuCl_2$ followed by a return to unpolluted saltwater for one week, signs of recovery and restoration of the erythrocyte population of the fish to normal shapes were evident; but the cells still showed signs of internal injury.

Electronic Printouts:

The BMA was not available to assay the fish exposed to $CuCl_2$ for 45 minutes.

Just as the photomicrographs gave a fairly normal picture of the cell condition at an exposure of 4.5 hours, so the BMA printout of this interval appeared quite normal (page 48). The peaks of the three triggering delay intervals all overlapped, and the 8μsec and 25μsec intervals both had well-defined secondary peaks. The printout of the 5 hour exposure was a markedly abnormal printout (page 48). These findings indicate that the effect varied with time among the individual fish.

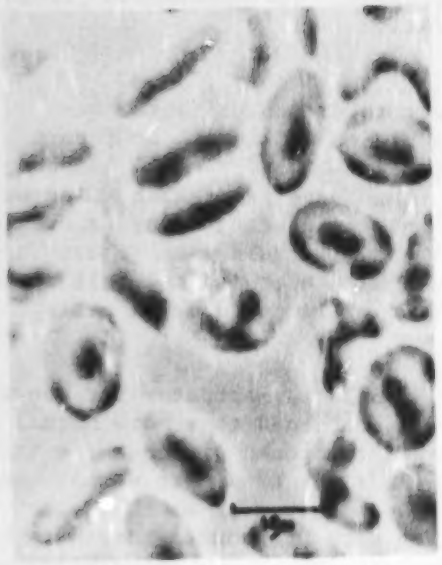
The BMA printout of the 24 hour exposure to $CuCl_2$ was abnormal. It correlated with the condition of the cells seen in the photomicrographs (page 51). The 4μsec triggering delay interval had two distinct peaks, the second being slightly larger than the first. The first peak appeared at channel 11, corresponding to an apparent diameter of about 3.4μ, the average width of the nuclei. As can be seen in the photomicrographs (page 49), there were few free nuclei; instead, the cytoplasm had broken down and was hugging the nuclei. Thus, since there was no swelling of the nuclei, the pulses generated corresponded to the width of the average nucleus. This peak was only present in the 4μsec interval because the cells were only as long as the nuclei and would not be seen in the 8μsec interval. The second peak of the 4μsec triggering delay intervalhad shifted upward on the scale to a point slightly larger than was usual. This peak probably represented the cell population whose cytoplasm had remained intact though swollen. The 8μsec interval had a peak similar to the second peak of the 4μsec interval; this indicated that the majority of the cells generating these pulses were fairly round or slightly oval. Since a similar peak was not seen in the 25μsec interval, these cells were probably not very long, but quite wide. The distribution seen in the 25μsec interval pointed to a segment of the population of erythrocytes of large and long cells; cells such as these were seen on the slides of the Wright stained cells.

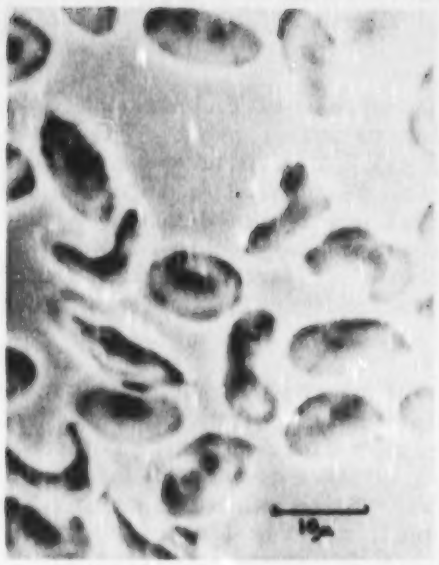
The electronic assay of the exposure to $CuCl_2$ for 44 hours was abnormal, but quite different from the printout of the 24 hour exposure test (page 51). The 4μsec triggering delay interval had lost the first peak that came up in the low channels. This may indicate that any free nuclei in the population were quite swollen; this was usually the way the nuclei appeared. The curve of the 4μsec interval was extremely broad indicating a great variation in the population sizes seen in this triggering delay interval. Usually the pulses observed at this interval were uniform, that was not the case here. In addition, there was a distinct population of very large cells as evidenced in the small secondary curve of this triggering delay interval. The peak of this interval was distinct from the 8μsec and 25μsec delay triggered peaks, and was one indicator of an abnormal cell situation. The peaks of the 8μsec and 25μsec triggering delay printouts had shifted quite far up scale indicating that a large segment of the population was swollen and rounded, a fact borne out by the photomicrographs. This was an abnormal printout with

characteristics seen often before - the separation of the peak of the 4 sec triggering delay interval from the primary peaks of the 8 sec and 25 sec triggering delay intervals; and the corresponding disappearance of the secondary peaks of the 8μsec and 25μsec intervals. The electronic printout showed a general swelling of the cell population.

A representative printout of the 10 hour return to saltwater was similar to the printout of the 44 hour exposure to $CuCl_2$ with no return (page 54). The broad 4μsec interval distribution with its small secondary peak was found here as it was in the 44 hour exposure test. The 4μsec delay interval was separate from the 8μsec and 25μsec triggering delay intervals, and the secondary peaks of the 8μsec and 25μsec intervals were gone. This was the usual pattern indicative of the abnormal copper-induced cell state. The electronic printout described a population of large, swollen cells.

Though the printouts of the return to unpolluted saltwater for one week were not normal, they indicated cells that had returned to a more normal size-shape distribution. Printout "a" showed the peaks of the three triggering delay intervals overlapping, one of the signs of a more normal condition. There was a slight suggestion of a secondary peak in the 8μsec interval, and the 25μsec interval had a much more pronounced secondary peak. Printout "b" showed the typical abnormal pattern in the peak separation and the disappearance of the secondary peaks in the 8μsec and 25μsec triggering delay intervals. But the hopeful sign here was that the peaks had shifted down scale indicating the absence of large swollen cells seen before. The printouts revealed the same preliminary signs of recovery seen in the photomicrographs (page 54).

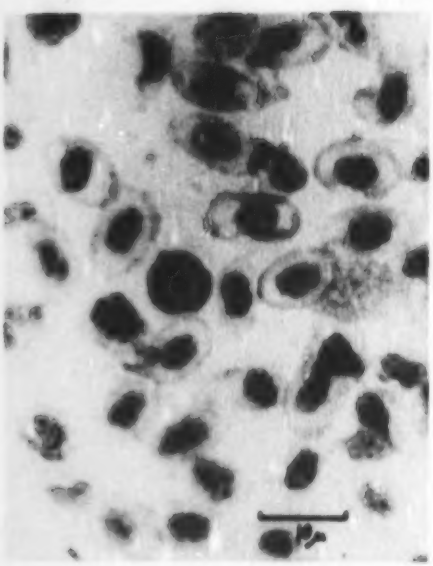


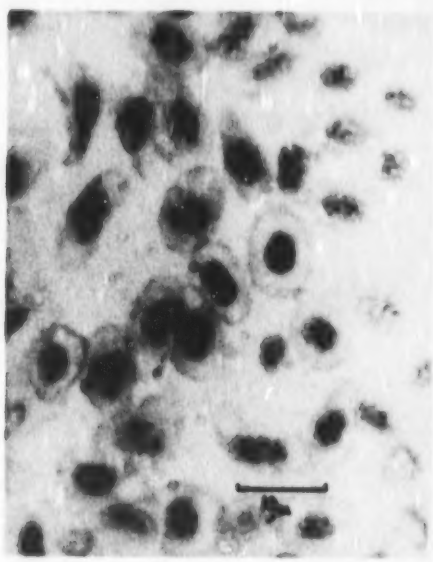


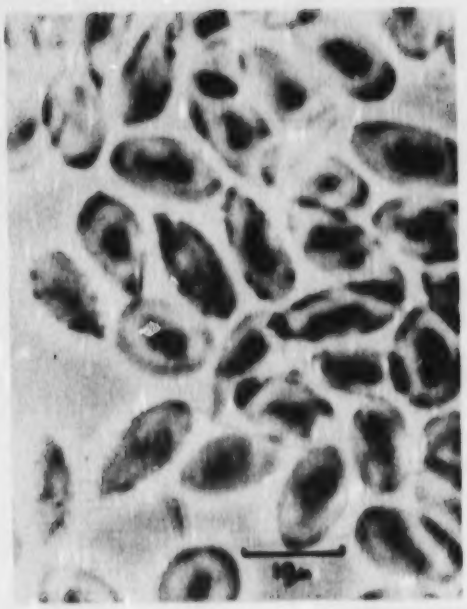
PHASE

$CuCl_2$-5ppm 45 mins: 5/22/73

STAINED

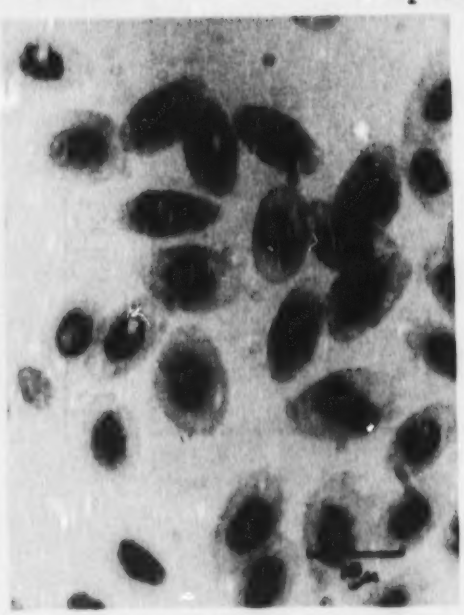


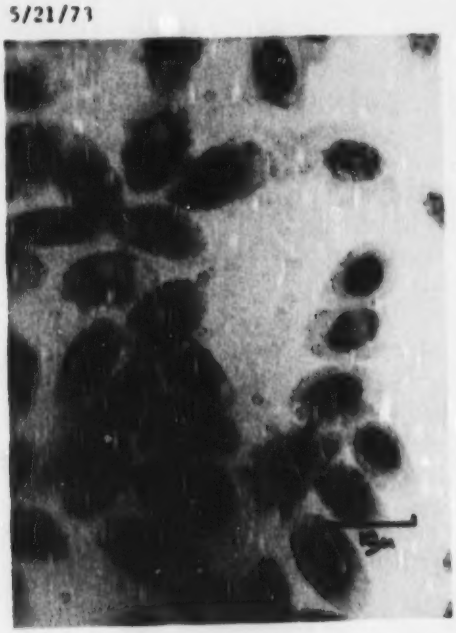




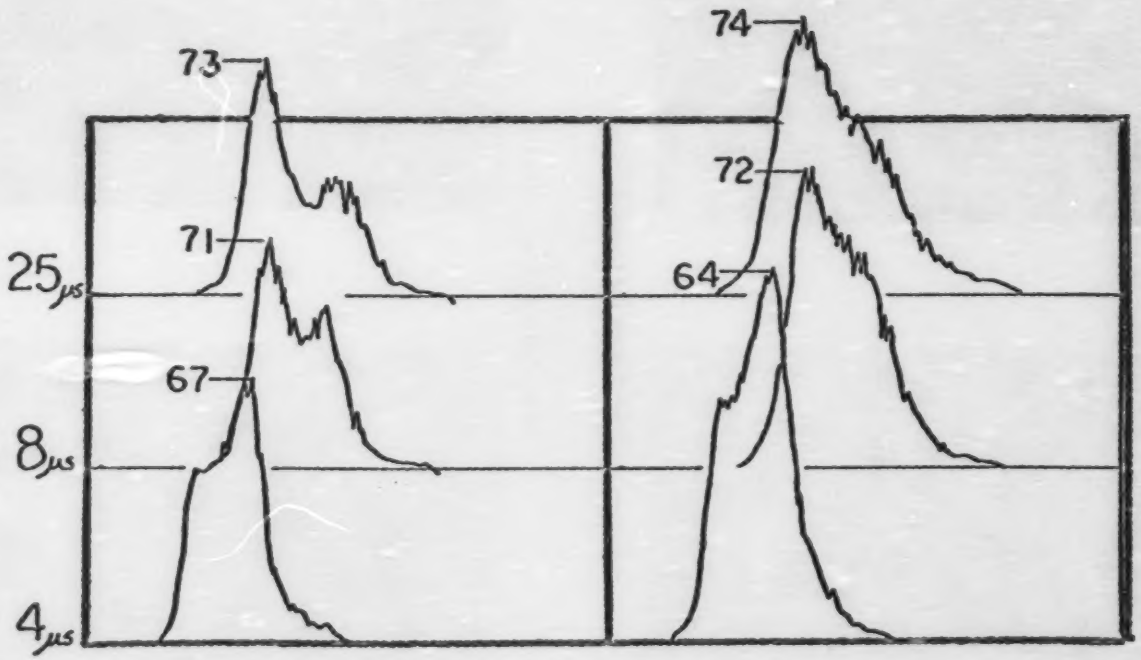
PHASE $CuCl_2$-5ppm 1 hr: 5/15/73

STAINED $CuCl_2$-5ppm 4.5 hr: 5/21/73





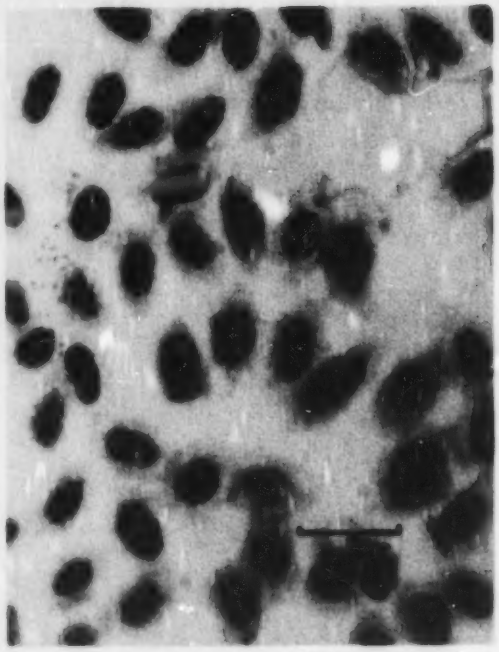
$CUCL_2$ – 5PPM

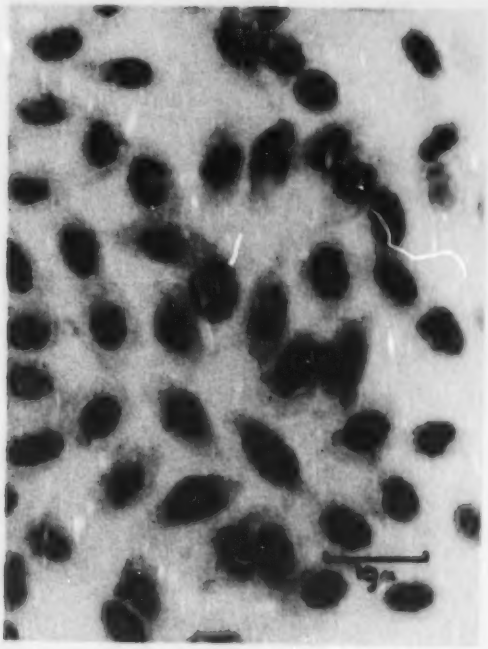


PHASE

$CuCl_2$-5ppm 24 hrs:5/22/73

STAINED

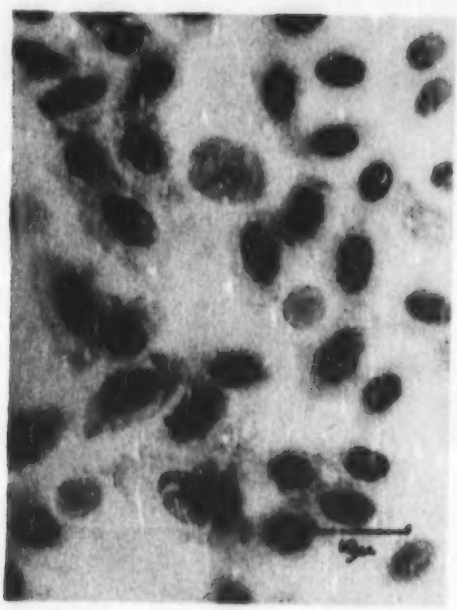




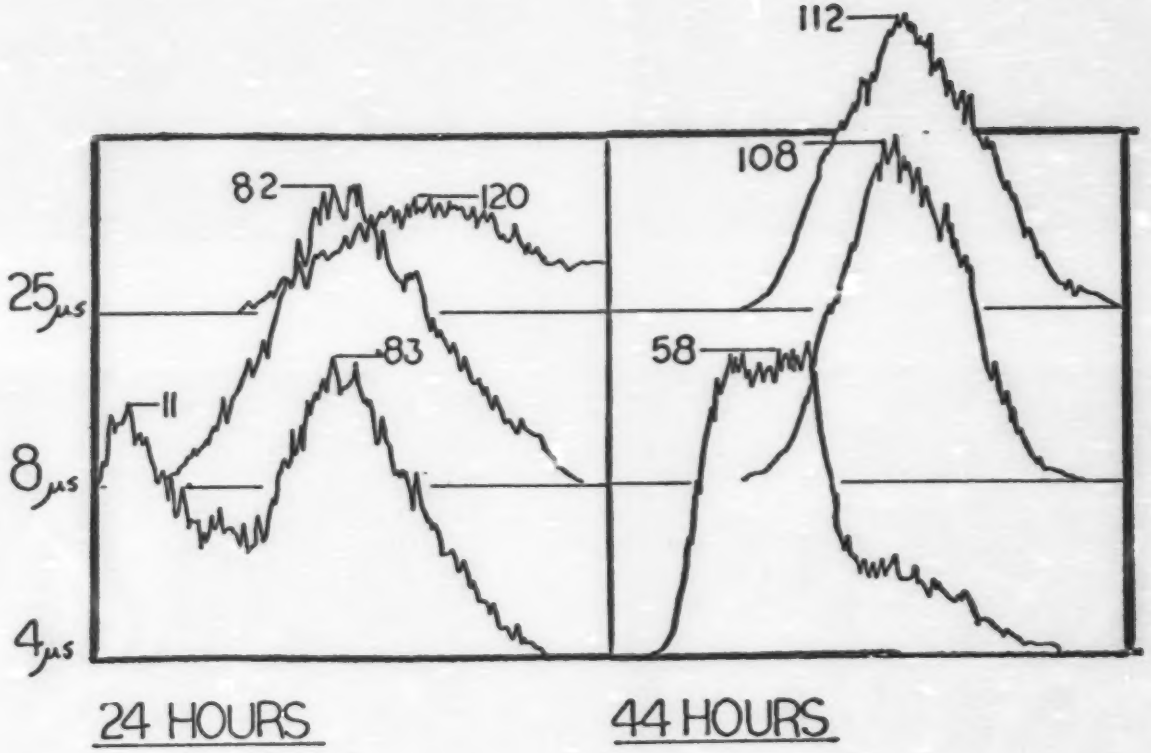
PHASE

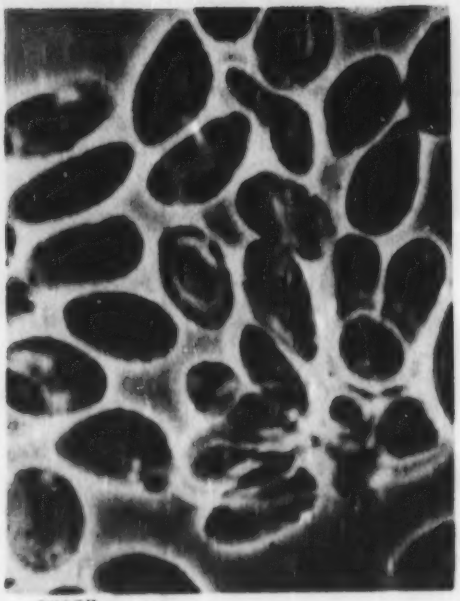
$CuCl_2$-5ppm 44 hrs: 5/29/73

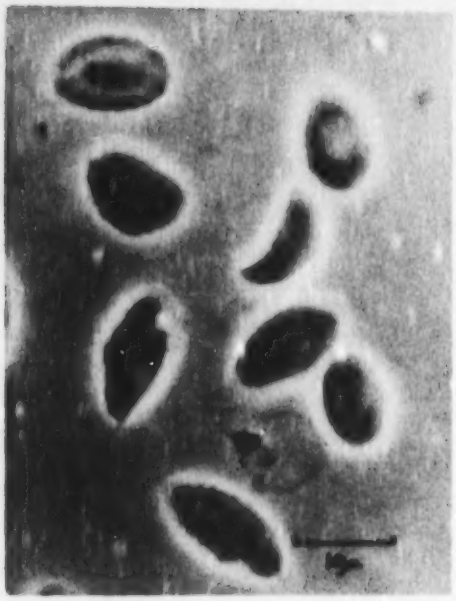
STAINED



$CuCl_2$ - 5PPM





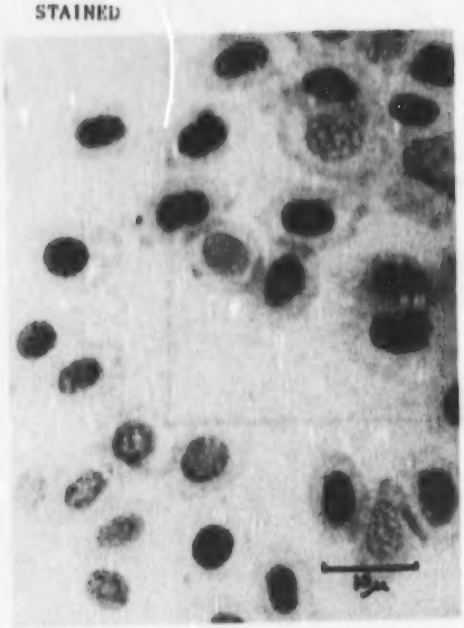


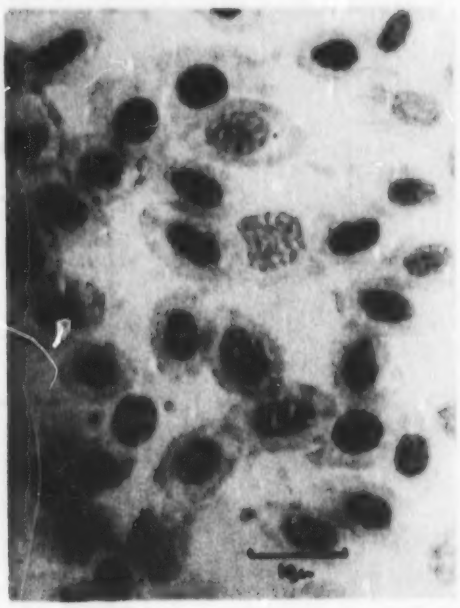
PHASE

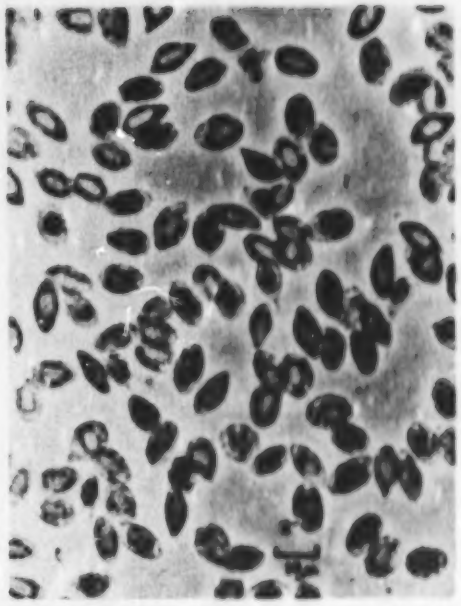
$CuCl_2$-5ppm 24 hrs: 5/29/73

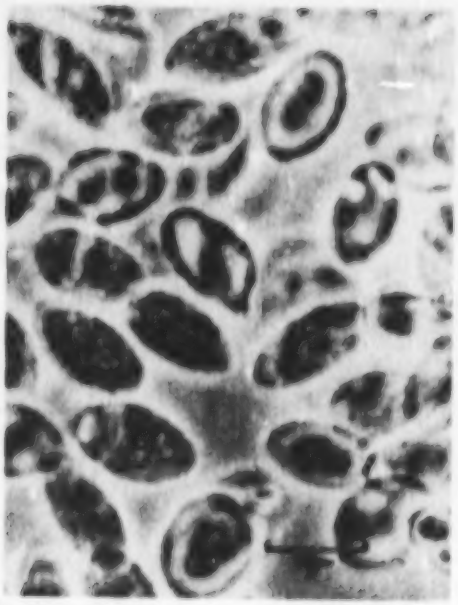
RETURNED SALTWATER: 10 hrs

STAINED









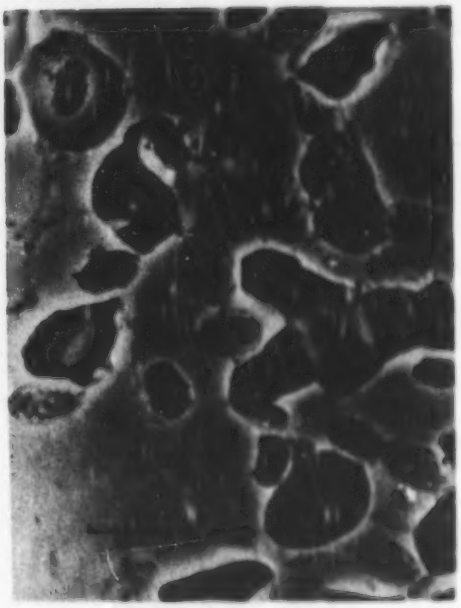
HIGH DRY

$CuCl_2$-5ppm 24 hrs: 6/4/73

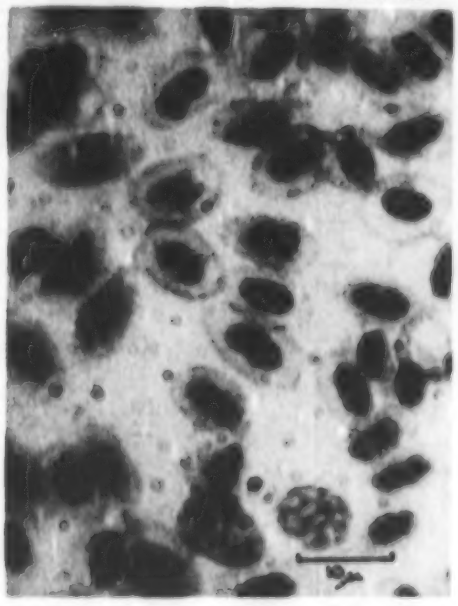
PHASE

RETURNED SALTWATER: 1 Week

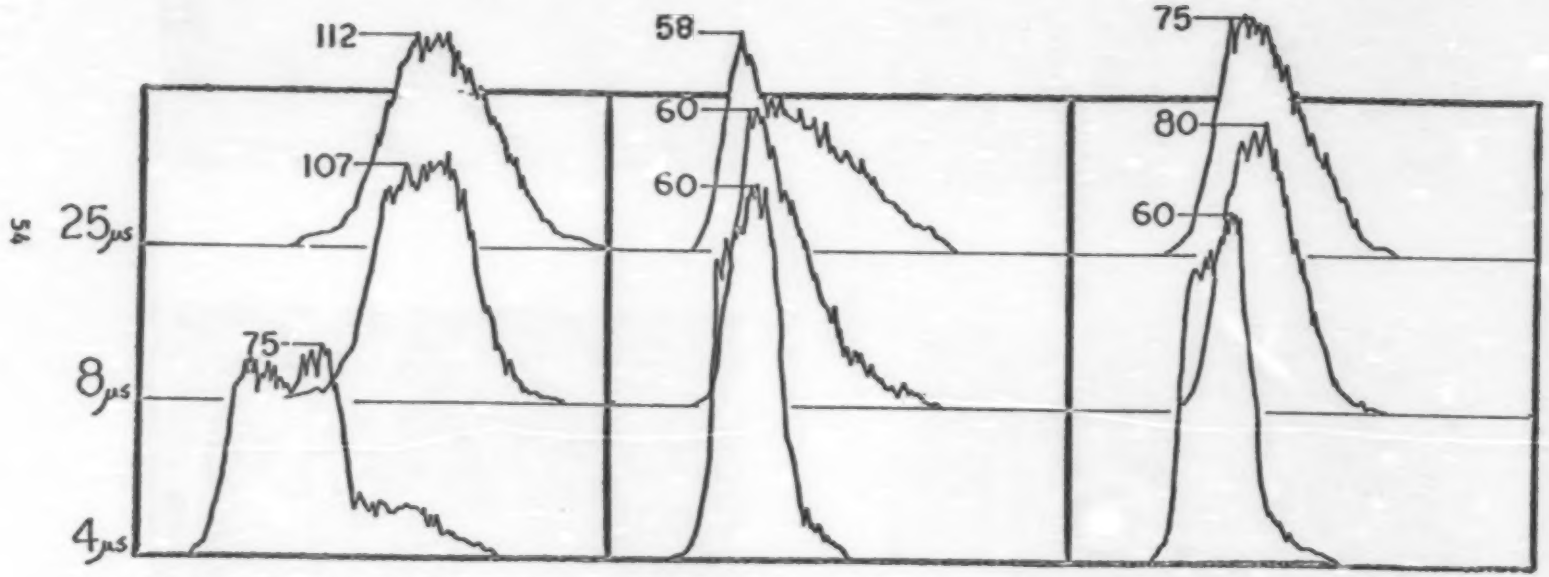
PHASE



STAINED



$CuCl_2$ 5PPM 24HRS: RETURN SALTWATER



RETURN: 10 HRS

RETURN: 1 WEEK

RETURN: 1 WEEK

DISCUSSION

Electronic Assay of Circulating Blood

In detecting changes in the normal cell state, the electronic printouts were examined in terms of changes in the distributions for the three triggering delay intervals, and how these three distributions related to one another. One aspect studied was how changes in one distribution related to changes in the other two. Further, these changes in the printouts were related to changes in cell membrane rigidity and changes in the general composition of the circulating blood.

Fish showing a normal cell state had electronic distributions in which the 4μsec triggering delay interval was one sharp peak; the 8μsec triggering delay interval was broader and had a smaller secondary peak following the first principal peak; and the 25μsec triggering delay interval was the broadest and, like the 8μsec interval, had a primary peak followed by smaller secondary peak. The major peaks of the three triggering delay intervals normally occurred in the same channel, indicating a large portion of the population being similar in size and shape.

Abnormal conditions were represented by the reduction or absence of the secondary peak of the 8μsec triggering delay interval. There was a corresponding peak separation between the 4μsec and 8μsec intervals as the secondary peak of the 8μsec interval began to disappear. The greater the reduction of the secondary peak, the further apart the 4μsec and 8μsec intervals shifted. The distributions for the 25μsec triggering delay interval seemed to follow that for the 8μsec interval. The peaks of the 8μsec and 25μsec intervals did not shift apart. The secondary peak of the 25μsec interval rarely disappeared.

In general, the peak channel for the normal distributions of the 4μsec and 8μsec triggering delay intervals will differ by only five channels (0.17μ). As the distributions began to change, the average peak channel difference becomes 10 (0.35μ). When two distinct peaks could be observed, the channel difference was at least 12 (0.42μ), and shifts of up to 70 (2.45μ) channels have been observed. The peak channel differences seem to fall into one of two categories - those that have a channel difference of less than 15, and those that differ by 30 or more. There seem to be few middle values.

In terms of cell types, what does this represent? The classification of circulating blood components of fish is unclear. In this report, we have adopted Gardner's classification (7) of the formed elements of the peripheral blood of F. heteroclitus. This is a recent work specifically examining F. heteroclitus. The blood components described by Gardner include mature nucleated erythrocytes, thrombocytes, small and medium lymphocytes, and eosinophils. The predominant cell type is the erythrocyte. Thrombocytes, the second most common cell type, are found in three forms, 1) the prothrombocyte, 2) intermediate forms, and 3) the mature thrombocyte (the latter being the most common of the three). In fresh preparations, the erythrocytes often exhibited a slight tail, appearing tear-shaped or triangular; this is in contrast to the familiar oval shape found in the Wright stained preparations.

Thrombocytes have the ability to extend pseudopods and form cohesive thrombocytic networks. In fresh preparations, mature thrombocytes, with no extended pseudopods are large (10μ diameter) rounded or somewhat oval in shape; the intermediate forms are more elongate or spindle-shaped. The lymphocytes and eosinophils, making up a small percent of the circulating blood population, will not be discussed further.

The electronic analysis of these components carried out on the BMA translated the cells into a population of electronic pulses. The shape of the pulse indicated the size and shape of an individual cell; and the composite of these pulses are accumulated by the BMA.

Any change in the membrane rigidity or composition of the cell population would be reflected in the BMA printout. In the freshwater experiments, for example, several fish showed internal hemorrhaging. When blood from such fish was examined on the BMA, there was a large shift to the lower channel range in the 4μsec triggering delay interval distribution. This build up was found to represent a great number of thrombocytes; these included many intermediate forms. Free nuclei, which are in the same size range as thrombocytes, were also identified from the Wright stained photomicrographs. The peak shifting and secondary peak reduction seen in the printouts of the metal-stressed fish reflect the alteration in membrane configuration and rigor, as evidenced by both the fresh and fixed preparations examined microscopically.

The fact that fish adapted to freshwater showed a higher proportion of peak separations may indicate that this adaptation to freshwater may not be complete. The peak shifting may also indicate that slight disruptions of the blood cell state result in peak separation. If this is the case, then this may prove helpful in determining sublethal but deleterious levels of metal pollutants. The separation of the peaks with the corresponding secondary peak reduction may be the first indication of an abnormal cell state.

DISCUSSION

Fundulus heteroclitus is a euryhaline fish capable of adjusting to a wide range of osmotic conditions. During the time we conducted freshwater adaptation studies with F. heteroclitus, we observed a pattern of behavior in response to the stress of the environment. The more abrupt the change in ambient salinity, the more intense the reactions were. On introduction to the new environment, the fish would remain motionless either on the bottom of the tank or at the surface of the water; some fish would swim on their side. A certain number of the fish would float belly-up indicating that death would soon follow. The more gradual the introduction to a 0‰ salinity environment, i.e. periods of time spent in tanks of 14 ‰ salinity, the less intense were their reactions. Further, the mortality dropped from 95%, which occurred when the fish were moved from 20‰ salinity to 0‰ salinity directly, to about 5% mortality, which occurred when the fish spent a minimum of one week in the 14‰ salinity tank before being put in 0‰ salinity.

Freshwater adapted fish used in metal pollutant tests were less able to adjust to the new experimental situation than test fish taken from the slatwater tank. The freshwater adapted fish appeared the worst affected by the metal insult. They were the most sluggish, did not eat when fed or seem to sense the food in the water; they were the first to die in the adverse conditions. What this may mean in a larger sense, is that even fish in the wild that may appear to be adapted to slightly deleterious conditions are less able to withstand any subsequent change in the environment. Such fish would be more susceptible to a lower level of pollutant in the water than would fish in a "healthy" environment.

Other researchers investigating the ability of Fundulus species to acclimate to changes in salinity found that these fish had a greater capacity for regulation against a dehydrating hyperosmotic medium than a hyposmotic one (9). Further, Garside (8) found that the upper lethal temperature that could be tolerated by Fundulus was lowest at 0‰ salinity, and highest at isosmotic salinity of 14‰. The acquisition and retention of osmotically active substances and the excretion of excessive H_2O seemed to create greater stress than the elimination of excess salts. This seems to agree with our findings of an increased vulnerability of the freshwater adapted fish when insulted by a metal pollutant.

The sublethal effects and mechanisms of actions of heavy metals on fish tissues are quite various and complex. Some of the often cited responses include congulation or precipitation of mucous on the gills and/or cytological damage to the gills. This creates a breakdown in gas exchange at the gills followed by hypoxia at the tissue level (2, 10). Another aspect of heavy metal poisoning is a neurotoxic phenomenon which affects the olfactory and lateral-line systems (6). Heavy metal ions are potent enzyme inhibitors; the toxicity of the metal cations is a function of their electron-attracting properties which combines them with the functional groups particularily the sulfhydral groups on enzymes (1, 3).

Our tests deal only with the circulating blood cells and their impairment. A model is presented which attempts to explain the response of the blood cell population to an insult by a metal, in this case, copper.

Throughout the experiments using copper as the heavy metal pollutant, a definite progression of responses had been observed. The first effect of the metal appeared to be on the membrane and involved a change from cells having a smooth surface to one in which the surface of the cell became jagged and irregular. The normal integrity of the membrane appeared to be lost and the cell shapes became quite irregular. These responses were noticed even after a 45 minute exposure to copper.

Rothstein (15) has reported that heavy metals at low levels change the permeability properties of membranes which alters the chloride equilibrium between erythrocytes and plasma. The loss of membrane rigor as observed in the sickling phenomenon, the irregularity of shapes and the serrated edges observed in our studies may be the result of an osmotic imbalance caused by such membrane property changes.

Other investigators have found erythrocyte membrane proteins which are contractile proteins, similar to actin and myosin (13). Changes in size and membrane deformability have been reported to be dependent on the conformational state of a membrane fibrous protein (14). This protein is controlled by intracellular ATP and Ca^{++} levels. When Ca^{++} levels are increased, both a conformational change of the membrane (contraction) and a decrease in membrane permeability are observed. Sr^{++} and Br^{++} have the same effect as Ca^{++} in causing conformational changes of the membrane. On the basis of these results it would seem highly reasonable that Cu^{++} would have the same effects on the red blood cell membrane as those produced by the other divalent cations (12). Thus, Cu^{++} would cause an alteration in the membrane's configuration and a loss of "rigidity"; allowing the red blood cell to assume numerous abnormal shapes.

After 24 hours exposure to $CuCl_2$ (5ppm), vacuoles or circular white clearings became evident in the cytoplasm of the erythrocytes. These clearings persisted in both the longer exposures to $CuCl_2$ and in the short-term reversal tests. Generally, one or two clearings were found in a cell. The cause or function of these clearings has not been determined. They may be indicative of an erythrocyte that is no longer viable (11). Similar phenomena were reported in the renal epithelium of F. heteroclitus, and the same effect was observed in the gills (5).

The nuclei showed the effects of the exposure to 5ppm $CuCl_2$ after the membrane became visibly affected. The initial reactions seen in the nuclei were the loss of the central location in the cell. The nuclei became lobed. The Wright stain was taken up unevenly with some of the nuclei apppearing quite opaque. As the cytoplasm began to break down, the nuclei started to swell, in some cases becoming nearly twice their normal size. In the phase contrast studies, at the longer exposures to 5ppm $CuCl_2$, the nuclei began to show the clearings or vacuolations seen in the cytoplasm.

Restoration to unpolluted saltwater for 10 hours after a 24 hour exposure to 5ppm $CuCl_2$ did not restore the normal erythrocyte shapes; prolongation of the return to the normal slatwater environment for one week still did not produce normal-shaped cells. Our observations of the cells which had had a one week restoration in normal saltwater after 24 hours in 5ppm $CuCl_2$ showed indications of a return to a more normal state. Whether this reversal was due to new cells being put into circulation rather than a reversal of existing cells, however, was not clear. In the phase contrast studies, there was a disappearance of the sickle shapes and irregular margins. The plasma membrane injuries seemed less severe. There were still internal vacuolations present, and the nuclei still showed some transparency. In the Wright stained cells, the cytoplasmic membrane appeared to have regained some of its original rigor; though the membrane still showed jagged, irregular edges. The nuclei were centrally located and took up the stain evenly.

An increase in the thrombocytic series was observed in the blood of F. heteroclitus after the specimen had been 1) placed in freshwater from a saltwater tank, 2) placed in unpolluted saltwater for 10 hours after 24 hours in 5ppm $CuCl_2$, and 3) placed in unpolluted saltwater for one week after 24 hours in 5ppm $CuCl_2$. Dawson (4) reported a similar thrombocytic response in the catfish after several weeks exposure to lead. In the phase contrast studies of fresh blood of F. heteroclitus, thrombocytic networks were observed after the specimens from the two recovery experiments were sacrificed. Thrombocytes were observed with extruded cytoplasmic pseudopods bound together by the cohesive property of their cytoplasm with erythrocytes caught in the mesh. Gardner and Yevich (7) in their studies of blood morphology of Cyprinodontiform fishes observed in some freshwater preparations an intricate thrombocytic network which they indicated served to function in the blood clotting process. Their studies concerned the normal blood and the phenomenon was observed in the fresh preparations when air was trapped beneath the coverslip. In our studies, this phenomenon was only observed under the environmental conditions stated above, and care was taken to obviate the possibility of air under the coverslip of all slides. The phenomenon is suggestive of an environmental disturbance in the blood due possibly to the copper.

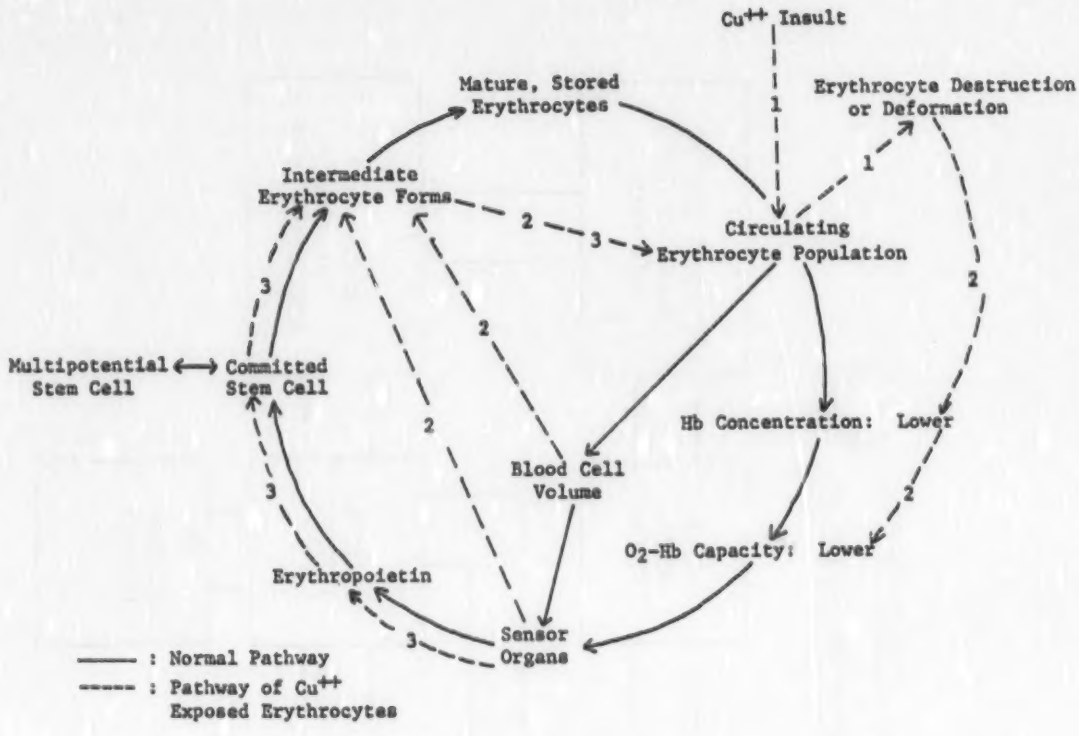
Observations were made on the viscosity and color of the blood at the time of sacrifice and when the blood was transferred to the slides in heparinized capillary tubes. The blood of specimens tested in freshwater, tested in a copper environment for 24 hours or longer, and tested in the reversal experiments was much darker than normal; this blood was a dark purple-red instead of the bright red of normal blood. This phenomenon of dark blood suggests that less oxygen is getting into the blood, or that there is a lowered oxygen acceptance capability of the blood. Further, as the blood was transferred to the slides, there was a decided resistance to flow which increased as the exposure to copper increased. Hughes (10) has suggested that interference with the gas exchange at the gill surfaces may result in tissue hypoxia. He suggests that a reduction in erythrocyte flexibility, effectively increasing the blood viscosity, and changes in cell volume are associated with increases in blood CO_2 levels.

Granulated erythrocytes were found in the circulating blood of fish exposed to 5ppm $CuCl_2$ for 24 hours and then returned to unpolluted saltwater for 10 hours. An identical phenomenon has been recorded by Gardner and Yevich (7) in teleosts undergoing increased erythropoietic activity in early summer. The phenomenon may be similar to that found in human blood production when under conditions causing increased erythropoiesis. Immature red blood cells are released directly into the circulation. This phenomenon happens either because of an increased call by the organism to make up for a loss of functional erythrocytes in the circulation or because of an alteration in the normal red blood cell production mechanism.

The results of our investigations in conjunction with the findings of other researchers indicate a complex series of reactions are taking place within fish sublected to copper ions in their environment. It appears that a rapid effect (Less than 2 hours) takes place in the circulating blood cells of the insulted fish. This is followed by a period of adaptation (4 to 6 hours) in which reserve cells are probably put into circulation to replace the cells disabled by the $CuCl_2$. Although it is not clear from the present data, it appears that once a blood cell is altered it recovery (return to normal appearance and function) is never complete. We have constructed a model which appears on page 61.

Our previous studies showed that in the case of F. heteroclitus, freshwater adaptation took approximately 16 days and that even then the fish were more susceptible to toxic insult. Our reversal studies indicate that the blood cell populations of fish receiving transient (24 hour) exposure to $CuCl_2$, although more normal than at the time of insult, are still partially aberrant. Since we are dealing with total populations of blood cells, it is not possible to know whether the changes we see are due to a reversal in the condition of the individual cells, or whether we are seeing a steady replacement of new unaltered red blood cells. The indications are that the latter is the case.

The final phase of the grant involved the design, calibration and delivery of an advanced fish blood analyzer. This was produced by the Grumman Health Systems (Woodbury, New York). Figure 5 shows a simple block diagram of the system, and Figure 6 shows a block diagram of the Grumman Health Systems Fish Blood Analyzer. One GHS Fish Blood Analyzer is presently in use at our laboratories and another is in use at the U.S. Water Quality Laboratories (EPA) in Narragansett, Rhode Island. Figure 7 is a photograph of the GHS Fish Blood Analyzer. A detailed instruction manual ("Fish Blood Analyzer Instruction Manual", Sias Medical Research Laboratories, 1973, pages 1-82) was delivered with the machine in November, 1973.



HYPOTHETICAL MODEL:

Effects of Cu^{++} Exposure on Fish Erythrocytes

Immediate:	(1)	Erythrocyte destruction or deformation resulting in an eventual decrease in either the effective erythrocyte volume or Hb-concentration.
5 - 10 Hrs	(2)	As a result of (1), a signal is received by the sensor organ which stimulates the release of stored erythrocytes and many intermediate erythrocyte forms into the general circulation in an attempt to maintain homeostatic balance.
Days or Weeks	(3)	As a result of (1), increased amounts of erythropoietin will be activated in an attempt to increase the numbers of stem cells that will differentiate toward erythrocytes. However, before any appreciable effects resulting from this pathway can be seen, a considerable period of time will pass.

FIGURE 5

BLOCK DIAGRAM OF MULTICHANNEL

ANALYSIS SYSTEM FOR FISH BLOOD

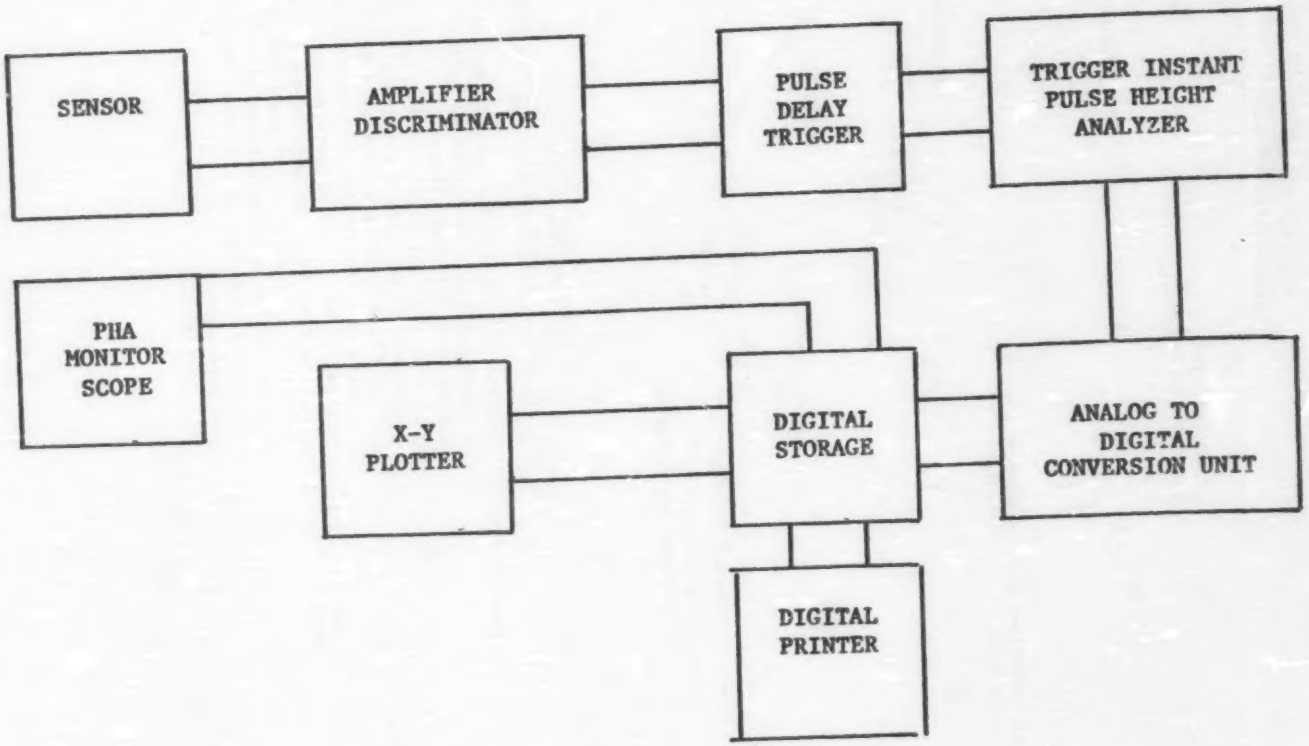


FIGURE 6

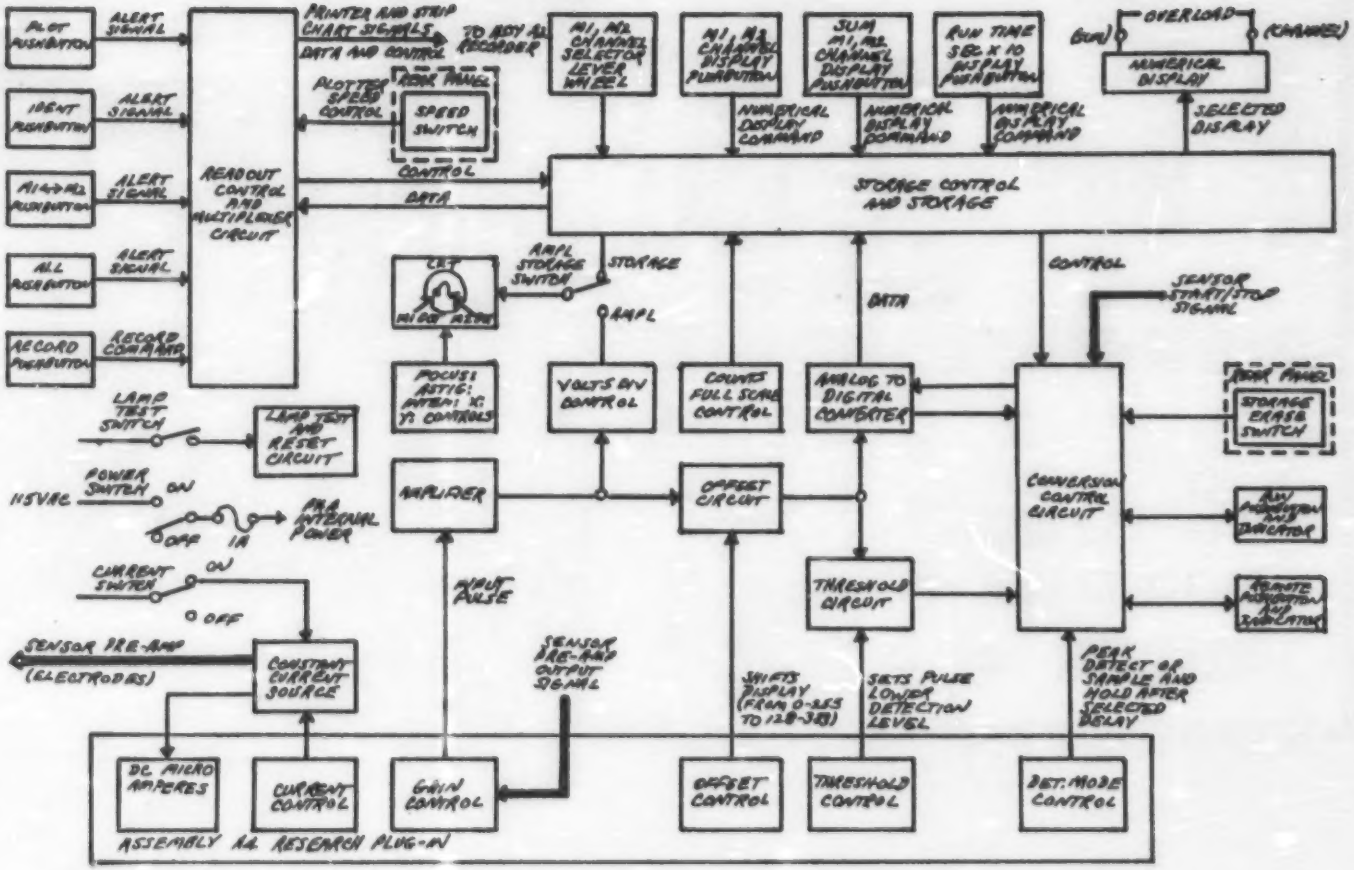
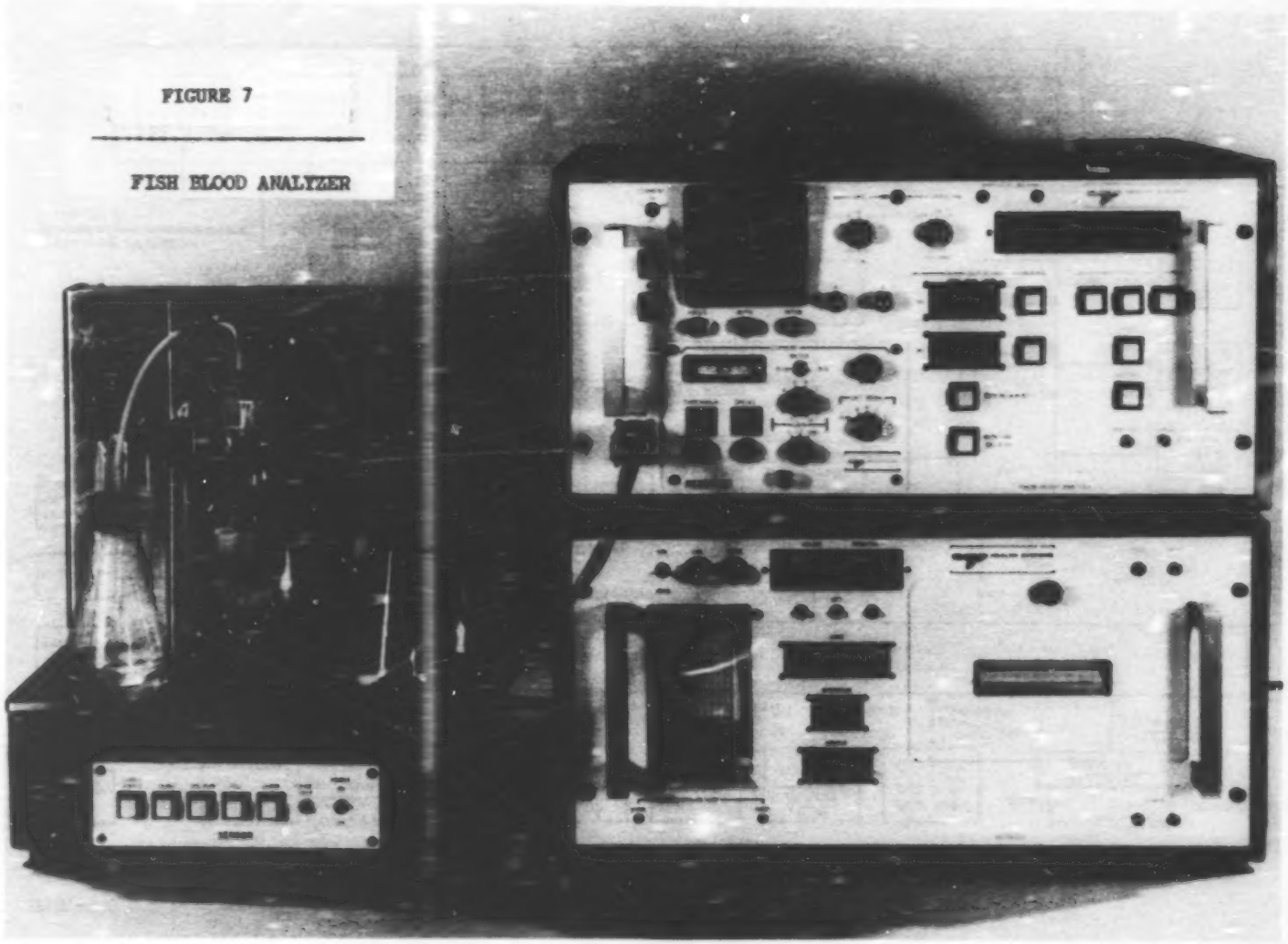

PLOT PUSHBUTTON
ALERT SIGNAL
IDENT PUSHBUTTON
ALERT SIGNAL
A1 AND A2 PUSHBUTTON
ALERT SIGNAL
ALL AGENTS PUSHBUTTON
ALERT SIGNAL
RECORD PUSHBUTTON
RECORD COMMAND
READOUT CONTROL AND MULTIPLEXER CIRCUIT
PRINTER AND STRIP CHART SIGNALS
DATA AND CONTROL
TO ASSY A2 RECORDER
PLOTTER SPEED CONTROL
FRONT PANEL
SPEED SWITCH
A1, A2 CHANNEL SELECTOR LEVER SWITCH
A1, A2 CHANNEL DISPLAY PUSHBUTTON
SUM A1, A2 CHANNEL DISPLAY PUSHBUTTON
RUN TIME SEC X 10 DISPLAY PUSHBUTTON
GUN
OVERLOAD
CHANNEL
NUMERICAL DISPLAY
SELECTED DISPLAY
NUMERICAL DISPLAY COMMAND
NUMERICAL DISPLAY COMMAND
NUMERICAL DISPLAY COMMAND
CONTROL
DATA
STORAGE CONTROL AND STORAGE
CRT
HIGH VOLTAGE
AMPL STORAGE SWITCH
STORAGE
AMPL
CONTROL
SENSOR START/STOP SIGNAL
DATA
FOCUS ASTIG ASTIG ADJ X, Y CONTROLS
VOLTS/DIV CONTROL
COUNTS FULL SCALE CONTROL
ANALOG TO DIGITAL CONVERTER
CONVERSION CONTROL CIRCUIT
FRONT PANEL
STORAGE ERASE SWITCH
LAMP TEST SWITCH
LAMP TEST AND RESET CIRCUIT
POWER SWITCH
115VAC
ON
OFF
FOR INTERNAL POWER
AMPLIFIER
OFFSET CIRCUIT
RUN INDICATOR AND TERMINATOR
REMOTE ACQUISITION AND TERMINATOR
CURRENT SWITCH
ON
OFF
INPUT PULSE
THRESHOLD CIRCUIT
SENSOR PRE-AMP
(ELECTRODES)
CONSTANT CURRENT SOURCE
SENSOR PRE-AMP OUTPUT SIGNAL
SHIFTS DISPLAY (FROM 0-255 TO 128-383)
SETS PULSE LOWER DETECTION LEVEL
PEAK DETECT OR SAMPLE AND HOLD AFTER SELECTED DELAY
DC MICRO AMPERES
CURRENT CONTROL
GAIN CONTROL
OFFSET CONTROL
THRESHOLD CONTROL
DET. MODE CONTROL
ASSEMBLY A2 RESEARCH PLUG-IN

FIGURE 7

FISH BLOOD ANALYZER



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